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VASCULAR REPAIR MECHANISMS EXPERIMENTAL, PHYSIOLOGICAL AND CLINICAL STUDIES

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To my teachers

ABSTRACT

Cardiovascular disease is the leading cause of global mortality and physical disability mainly due to the complications of atherothrombosis such as myocardial infarction or stroke. Physiological healing reaction takes place in the diseased vessel wall aimed to repair the vessel after an injury, noxious stimuli or altered physical forces. It plays a central role in such diverse conditions as in the formation of the fibrous cap in atherogenesis, in the repair of vulnerable lesions after plaque rupture, in restenosis after arterial interventions and in venous by-pass grafts. In this thesis, we investigated the mechanisms of vessel wall repair in various models. Molecular mechanisms involved in intimal hyperplasia were studied with a focus on the role of insulin-like growth factor-1(IGF-1) in smooth muscle cell (SMC) proliferation. Pharmacotherapy specifically targeting the IGF-l axis attenuated intimal hyperplasia after balloon injury through inhibition of SMC proliferation. Not only molecular signals from blood but also physical forces reach SMCs especially those which form the neointima. We demonstrated that increased levels of shear stress downregulate SMC proliferation and significantly alter gene expression. Tissue factor pathway inhibitor 2 is strongly upregulated by fluid shear stress in SMCs and can inhibit proliferation of both endothelial cells and SMCs. This implies that hemodynamic forces can directly effect SMC gene expression and in this way regulate intimal repair. We evaluated non-invasive ultrasound biomicroscopy technique and found it sufficient to accurately monitor the healing reaction of the injured artery and to assess an effect of pharmacological inhibition of neointima formation. In human atherosclerotic lesions, expression of a limited number of genes previously described to be involved in plaque stability were analyzed with respect to clinical variables assumed to reflect lesion phenotype. We showed that symptoms, statin treatment, and ultrasound morphology were clinical markers of plaque stability while time between the last qualifying symptom and surgery correlated with patterns of plaque healing. By increasing our understanding of the molecular pathways that regulate vessel wall repair, we can develop pharmacological methods to control SMC activation and proliferation to improve outcome after vascular surgery. Additionally, the ability to monitor and pharmacologically regulate vessel wall healing could provide possibilities to combat plaque instability and prevent the clinical consequences of atherothrombosis.

LIST OF PUBLICATIONS

This thesis is based on the following original articles and manuscripts, which will be referred to in the text by their roman numerals:

I. **Razuvaev A**, Hederson B, Girnita L, Larsson O, Axelson M, Hedin U, Roy J.
The cyclolignan picropodophyllin attenuates intimal hyperplasia after rat carotid balloon injury by blocking insulin-like growth factor-1 receptor signaling.
Journal of Vascular Surgery, 2007 July; 46(1): 108-15

II. **Razuvaev A**, Lund K, Roy J, Hedin U, Caidahl K.
Noninvasive real-time imaging of intima thickness after rat carotid artery balloon injury using ultrasound biomicroscopy.
Atherosclerosis, 2008 August; 199 (2): 310-6

III. **Razuvaev A**, Ekstrand J, Folkersen L, Marcus D, Swedenborg J, Hansson G, Gabrielsen A, Paulsson-Berne G, Roy J, Hedin U.
Correlations between clinical variables and gene expression profiles in carotid plaque instability.
Manuscript

IV. Ekstrand J, **Razuvaev A**, Roy J, Hedin U.
Expression of issue factor pathway inhibitor 2 is induced by fluid shear stress in vascular smooth muscle cells.
Manuscript

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LIST OF ABBREVIATIONS

AF	amaurosis fugax
BiKE	Biobank of Karolinska Carotid Endarterectomies
BrdU	bromodeoxyuridine
CT	computer tomography
DES	drug eluting stent
DWI	diffusion-weighted MR-brain–imaging
EC	endothelial cells
ECM	extracellular matrix
ERK	extracellular signal – regulated kinase
FGF	fibroblast growth factor
FSS	fluid shear stress
GSM	grey scale mean
IEL	internal elastic lamina
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IGFBP	IGF binding proteins
IL	interleukin
IMT	intima-media thickness
IRS	insulin receptor substrate
IT	intima thickness
IVUS	intravascular ultrasound
MAPK	mitogen-activated protein kinase
MMP	matrix metalloprotease
MRI	magnetic resonance imaging
MS	minor stroke
MT	media thickness
PCA	percutaneous coronary angioplasty
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PET	positron emission tomography
PI3K	phosphatidyl inositol 3-kinase
PPP	picropodophyllin
PTA	percutaneous transluminal angioplasty
SMC	smooth muscle cell
TEA	thromboendarterectomy
TFPI-2	tissue factor pathway inhibitor 2
TGF	transforming growth factor
TIA	transient ischemic attack
TNF	tumor necrosis factor
UBM	ultrasound biomicroscopy

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Cardiovascular disease is the leading cause of global mortality (WHO, fact sheet N317, 2007). Every third adult in Western countries has some form of cardiovascular disorder. The Western world has had a high cardiovascular disease prevalence due to increasing life expectancy and life style alterations since many years (Rosamond, Flegal *et al*. 2008). However, during the past decades developing countries such as Russia and Turkey witnessed a dramatic increase in the prevalence of atherosclerotic vascular disease and its mortality (Averina, Nilssen *et al*. 2003; Tokgozoglu and Baris Kaya 2008). The major clinical manifestations of atherosclerosis are related to reduction in blood flow through coronary, precerebral or peripheral arteries. Atheroma formation starts at a young age with the accumulation of lipids in the intimal layer of large and medium sized arteries. This is followed by initiation of an inflammatory response that leads to plaque growth and lumen loss. In parallel with atheroma progression, a healing process occurs within the plaque. This course includes activation of smooth muscle cells, which by forming the fibrous cap over the lesion, keep the thrombogenic content of the lesion away from blood flow. When this process is not sufficient, the plaque may rupture and cause thrombosis of the artery. Acute flow interruption by atherothrombosis leads to life threatening conditions such as myocardial infarction or stroke and will also cause physical disability in many survivors (Donnan, Fisher *et al*. 2008). As formation of an atherosclerotic plaque takes place over decades, treatment possibilities at the time of clinical manifestation are limited. Current therapeutic strategies are oriented towards the prevention of acute atherothrombotic events through a range of therapeutic regimens, which address adverse risk factors for cardiovascular disease. A large portion of coronary events, such as myocardial infarction, may be preventable through adherence to healthy lifestyle practices (Chiuve, McCullough *et al*. 2006). Antiplatelet therapy has been shown to decrease risk for acute cardiovascular events. Lipid-lowering therapy is used to slow down progression of atherosclerotic changes in the vessel wall. In addition to the improvement of lipid metabolism a class of drugs that lower cholesterol levels ,statins, can suppress inflammatory activity in atherosclerotic lesions and therefore improve prognosis for people with cardiovascular disease. In many situations, medical treatment is not enough to prevent acute events or improve blood

flow through a stenosed part of the artery and surgical intervention is required. Surgical procedures include removal of the plaque, creation of a bypass or endovascular dilation of the artery. However, surgical intervention causes an iatrogenic injury of the vessel, which triggers a healing response. This process can in some cases cause restenosis, a reduction of the lumen by extensive thickening of the wall – intimal hyperplasia.

The biology of cardiovascular disease is complex and despite substantial progress in the field, it is far from being completely understood. Better understanding of healing processes in the vessel wall will lead to development of new strategies in prevention and treatment of atherosclerosis and its complications.

1.2 ATHEROSCLEROSIS

1.2.1.1 Natural history of atherosclerotic lesions

Atherosclerosis is a diffuse process that starts early in childhood and progresses asymptomatically through adult life. The process includes structural changes in the intima and media of large and medium-sized arteries, initially starting with in endothelial abnormalities and eventually resulting in atherosclerotic plaques. Later in life, it is clinically manifested as coronary artery disease (CAD), stroke and peripheral arterial disease (PAD).

Dysfunction of the endothelium is the earliest pathologic process of atherosclerosis, which occurs at sites in the arterial tree where laminar flow is disrupted and is involved in the recruitment of inflammatory cells into the vessel wall and lipid retention.

Atherogenic lipoproteins such as low-density lipoproteins (LDL) enter the intima, where they are modified by oxidation or enzymatic activity and accumulate in the extracellular space. At the same time, T cells and macrophages, triggered by endothelial dysfunction, enter the vessel wall, and interact in a synergistic manner. Autoreactive T cells recognize oxLDL, heat shock proteins and other antigens and locally release proinflammatory cytokines. Uptake of lipoproteins by macrophages leads to the generation of foam cells, which are laden with lipid. The accumulation of foam cells leads to the formation of fatty streaks, which are considered to be the initial atherosclerotic lesion.

The recruitment of monocytes to the intima and their subsequent differentiation into macrophages continues during the expansion and progression of the plaque. Macrophages have a wide range of activities, including intracellular accumulation of lipids and secretion of multiple chemokines, other cytokines and proteases that leads to attraction of other types of inflammatory cells and lesion progression (Nilsson and Hansson 2008). T cells are recruited to atherosclerotic intima accelerating the inflammatory state. Macrophage function is regulated by Toll-like receptors, which are pattern-recognition receptors that are involved in initiating innate immune responses. Other cell types like natural killer cells, mast cells, dendritic cells and platelets also have been implicated to atherosclerosis (Llodra, Angeli *et al*. 2004; Hansson 2005; Vanderlaan and Reardon 2005; Davi and Patrono 2007; Kovanen 2007).

Foam cells may die, resulting in the release of cellular debris and cholesterol. In parallel, smooth muscle cells (SMC) of the media become activated and migrate to the intimal layer. SMCs form a fibrous cap beneath the endothelium by proliferation and synthesis

of a collagen rich extracellular matrix (ECM), and this separates the plaque from the blood. Formation of fibrous cap by SMCs may be regarded as a healing response of the vessel wall where the cells engage the ongoing inflammation in the intima. A necrotic core is formed within the plaque and the lesions progress to fibroatheroma. While the atheroma develops, formation of new capillaries occurs in the plaque. These immature neovessels are fragile and contribute to progression of the lesion causing intraplaque hemorrhage (Virmani, Finn *et al*. 2009).

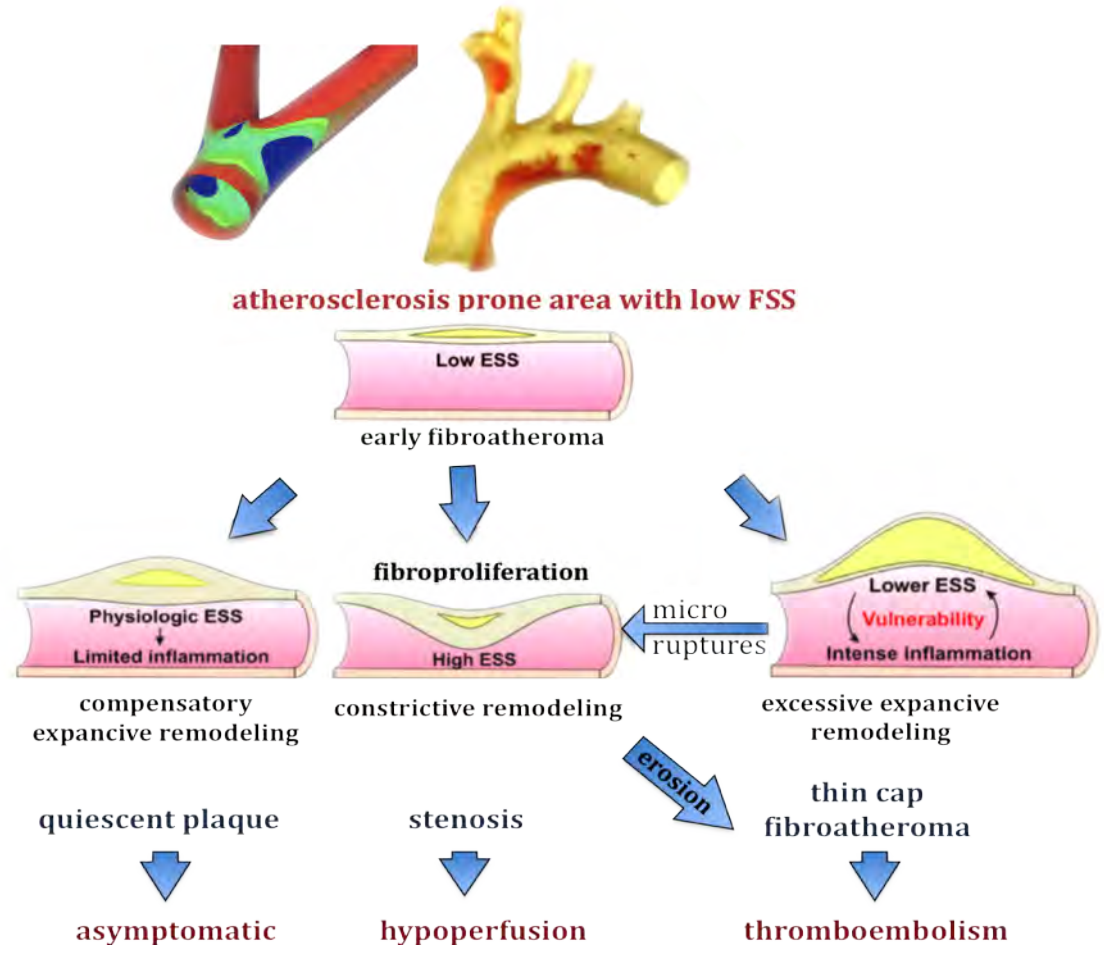


Figure 1. Development of atherosclerotic plaque in relation to fluid shear stress values (ESS = endothelial shear stress). Adopted from Chatzizisis YS, 2007, JACC.

As the plaque grows, compensatory remodeling takes place, so that the size of the lumen is preserved while its outer diameter increases. In this way, atherosclerotic lesions can progress without compromising the lumen (Glagov, Weisenberg *et al*. 1987). Importantly, lipid-rich lesions leading to acute coronary syndromes are often mildly stenotic, due to significant positive remodelling, and therefore are not detectable by angiography. As originally described by Glagov, positive remodeling can compensate lesion growth until the plaque area is over 40% of the lumen size (Glagov, Weisenberg *et al*. 1987).

Over the past several years, it has been recognized that plaque composition, rather than plaque size or stenosis severity, is important for plaque rupture and subsequent thrombosis. Ruptured plaques and plaques prone to rupture, tend to be large in size with associated expansive arterial remodeling, thin fibrous cap with a thick or large necrotic lipid core with immuno-inflammatory cell infiltration in the fibrous cap and adventitia, and increased plaque neovascularity, sometimes with intraplaque hemorrhage.

Such a non-obstructive plaque can rupture and the thrombogenic material of the necrotic core, including tissue factor, is exposed to the blood causing thrombosis. The large thrombus can block the lumen and cause acute ischemia. Alternatively, in larger vessels like the carotid artery, thrombus formation may not occlude the vessel but cause thromboembolism with flow interruption in smaller more distal vessels. Plaque disruption and subsequent thrombus formation is responsible for the onset of most acute coronary syndromes and strokes. Another mechanism of atherothrombosis is plaque erosion, a process related to the loss of endothelium either secondary to vasospasm or due to the inability of endothelial cells to adhere to an underlying matrix rich in hyaluronan (Farb, Burke *et al*. 1996; Kolodgie, Burke *et al*. 2002).

A large number of studies in the field of atherothrombosis have been focused on the mechanisms underlying plaque rupture. Rupture is associated with the degradation of collagen and elastin by extracellular proteases. Many members of the matrix metalloproteinase (MMP) family have been found in atherosclerotic plaques and have been suggested to be involved in rupture (Newby 2005). Another factor contributing to plaque rupture is abundant apoptosis of macrophages and SMCs (Clarke, Figg *et al*. 2006). Death of SMC leads to formation of a thin and weak fibrous cap, which is prone to rupture especially in the shoulder region where the cap is often thinnest and most heavily infiltrated with inflammatory cells (van der Wal, Becker *et al*. 1994). Vulnerability of an atherosclerotic lesion has largely been associated with enhanced inflammation (Shah 2003). The activity of inflammatory cells such as T-cells and

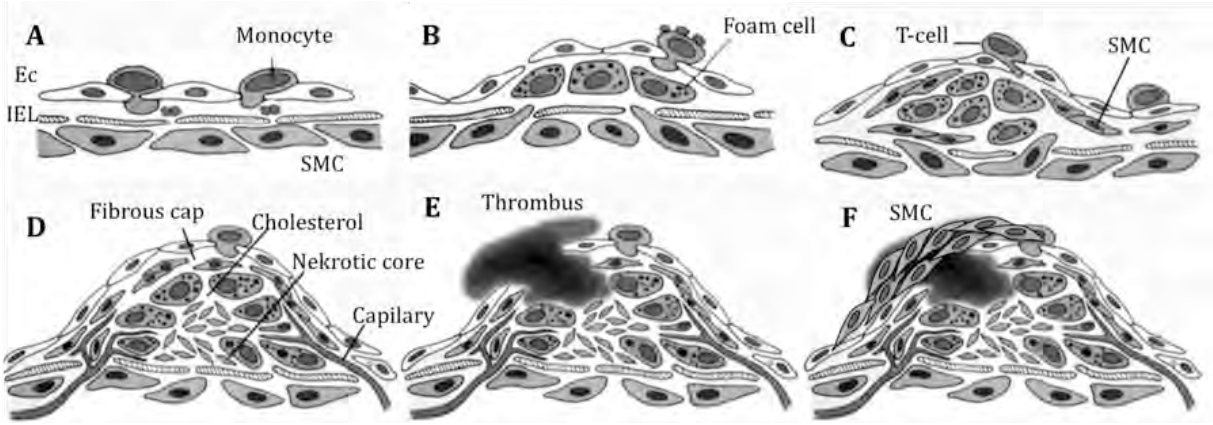


Figure 2. Natural history of atherothrombosis. A – lipid accumulation; B- Macrophage activation and formation of foam cells; C - SMC form a fibrous cap over the necrotic core; D – an advanced plaque with neovessels inside the necrotic core; E – plaque rupture with thrombus formation; F – formation of a new fibrous cap over the ruptured area.

macrophages, cytokine release, secretion of MMPs and other soluble factors, contribute to the thinning of the fibrous cap due to collagenolysis and apoptosis of SMCs (Libby 2000).

In parallel with destructive processes in the plaque, a healing response takes place, which is generally attributed to the activation of SMC. These cells are the main producers of ECM and responsible for the formation and maintenance of the fibrous cap (Burke, Kolodgie *et al*. 2001). Importantly, functional, viable medial SMCs are also crucial for arterial remodeling and prevent lumen loss and hypoperfusion. (Seo, Lombardi *et al*. 1997). In case, repair mechanisms are incapable to prevent atherothrombosis and the patient survives, an additional wave of healing activity is induced by this event. Activated by platelets SMCs proliferate and form a layer over the site of rupture and thrombosis with following restoration of the endothelium. This new fibrous cap separates again the necrotic core from the blood flow. Further proliferation of SMC and synthesis of ECM leads to the plaque progression. The newly formed fibrous cap over the rupture resemble a scar tissue and contain a proteoglycan rich matrix and highly ramified SMC and, later, dense collagen (Schwartz, Virmani *et al*. 2000).

Eventually, if the plaque does not rupture and the lesion continues to grow, it can compromise the lumen and result in blood flow reduction with following ischemia. Small ruptures, which do not cause occlusion of the vessel also contribute to lesion growth by formation of the scar – new fibrous cap over the organized thrombus.

1.2.2 Clinical manifestations of atherosclerosis

Atherosclerosis may cause chronic or acute ischemia of various organs. Most common are coronary heart disease (myocardial infarction and angina), PAD (intermittent claudication and gangrene) and cerebral ischemia (transient ischemic attack - TIA), ischemic stroke) (Gonzalez and Kannewurf 1998). Coronary thrombosis is a leading cause of mortality, and stroke is the leading cause of disability in adults, the second most important cause of dementia and the third most common cause of death in Western countries (Leys 2001). At the age between 55 and 74, myocardial infarction (MI) occurs very frequently and is a leading cause of disability in this age group. In contrast, in those aged 75 and over, stroke and TIA are more prevalent than MI and carotid artery stenosis is a frequent finding in the general population with a prevalence of 75% in men and 62% in women over 64 years as determined by ultrasonography in the Cardiovascular Health Study(O'Leary, Polak *et al*. 1992; de Rijk, Launer *et al*. 2000; Di Carlo, Launer *et al*. 2000; Lobo, Launer *et al*. 2000). The health burden from stroke is considerable.

Peripheral arterial disease has a prevalence of 10–20% in people over 60 years and is associated with significant mortality and moderate to severe functional impairment. It is defined by atherosclerotic obstruction of the abdominal aorta and arteries to the legs that reduces arterial flow during exercise and/or at rest, and is a common manifestation of systemic atherosclerosis. Intermittent claudication remains a major medical and socio-economic burden(Fowkes, Housley *et al*. 1991).

From the clinical point of view, atherosclerosis is seen as a single pathology that affects different vascular territories. TIA, intermittent claudication and unstable angina have clinical similarities as well as myocardial infarction, stroke and gangrene of lower limbs. There is also large overlap in prevalence between PAD, CAD and cerebrovascular disease (Criqui, Denenberg *et al*. 1997). Patients with PAD, even in the absence of a history of myocardial infarction or ischemic stroke, have approximately the same relative risk of death from cardiovascular causes as patients with a history of coronary or cerebrovascular disease. It has been shown that the risk of mortality is proportional to the severity of PAD (Newman, Shemanski *et al*. 1999). The three mentioned clinical entities share most of the risk actors such as hyperlipidemia, high fat diet, lack of physical activity, stress and diabetes (Sattar, Gaw *et al*. 2003).The risk factors for PAD are similar to those for CAD and cerebrovascular disease, but diabetes and cigarette smoking have a particularly strong association with PAD (Criqui 2001).

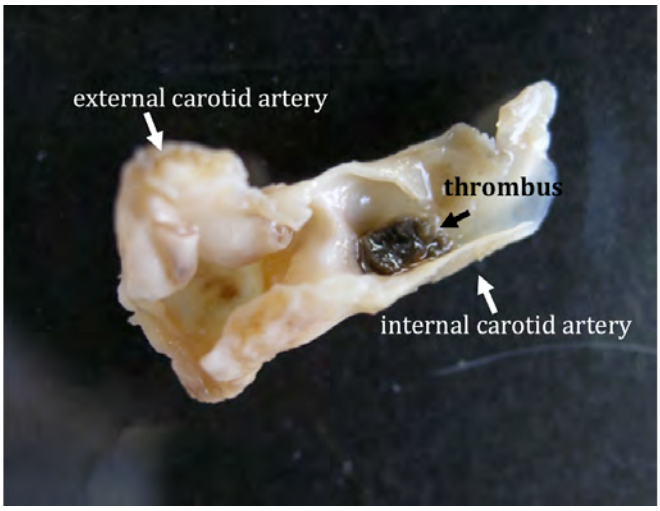


Figure 3. An atherosclerotic plaque obtained from a patient with symptomatic cerebrovascular disease. A thrombus was found in the internal carotid artery.

Despite the similarities, atherosclerotic lesions of different vascular beds have unique characteristics. Compared with most high-risk coronary plaques, high-risk carotid plaques are more stenotic, more heterogeneous and often fibrous. Plaque rupture is often related to an intramural hematoma or dissection (Glagov, Zarins *et al*. 1988). In contrast to coronary arteries, blood flow is significantly higher in carotid vessels and the lesion size is larger, which may be responsible for higher rates of distal embolisation that cause strokes or TIAs. Ruptures of the carotid plaques are characterized by seldom formation of a luminal thrombus, which is otherwise small and often leaves an ulcerated plaque in contrast to coronary lesions (Virmani, Finn *et al*. 2009). High-risk plaques of the lower extremities appear to be very stenotic and fibrotic (Ouriel 2001). The prevalence of hyperthrombotic state of the blood, caused by diabetes, cigarette smoking, and dyslipidaemia in PAD patients suggests that acute ischemia in the leg is associated with hyperthrombogenicity of the blood (Sambola, Osende *et al*. 2003). Relatively low blood flow velocity in lower limb arteries is another factor that differs from atherothrombosis at other localizations.

1.2.3 Prevention of thromboembolism

Some of the risk factors for cardiovascular disorders are modifiable and there is evidence that up to 80 % of acute ischemic events can be prevented by life style modification: correction of the diet, regular exercise and smoking cessation (WHO, fact sheet N317, 2007). For subjects with a high risk of atherothrombosis, additional medical treatment is required for primary prevention of thromboembolic events. For example, patients with PAD should be candidates for secondary prevention strategies including aggressive risk factor modification, antithrombotic treatment, lipid lowering and antihypertensive therapy. Medical therapy has been shown to have a considerable impact on the progression of atherosclerosis in all vascular beds including precerebral arteries. Treatment with statins significantly reduces stroke risk most likely by reducing plaque inflammation (Libby 2000; Baigent, Keech *et al*. 2005; Kunte, Amberger *et al*. 2008; Sillesen, Amarenco *et al*. 2008). However primary prophylaxis is not enough to prevent all cases of ischemia. It is also known that a significant number of acute MI and stroke occur in patients with no previous history of arterial disease, and therefore are not taking preventive measures.

1.2.3.1 Secondary prevention of stroke

A thromboembolic event is not only a dangerous condition, which requires acute measures, but in many cases also a sign of an unstable atherosclerotic plaque and therefore a high risk of a new embolisation. Patients with either transitory ischemic attack or minor stroke (MS) suffer a risk of approximately 5% within the first two weeks after the first event compared with approximately 2% risk of stroke annually in subjects with asymptomatic carotid stenosis (Giles and Rothwell 2007). For individuals with severe and symptomatic carotid lesions, carotid endarterectomy is recommended (Liapis, Bell *et al*. 2009). These recommendations are based on data from the major clinical trials, which addressed benefits and risks of carotid endarterectomy in different groups of patients. (Rothwell, Eliasziw *et al*. 2003; Coull, Lovett *et al*. 2004; Rothwell, Eliasziw *et al*. 2004). These studies were conducted about 10 years ago (except ACST - 2003) and included a considerable number of patients. In the mentioned trials, the degree of stenosis was used as a variable to predict risk of stroke and therefore severity of lumen loss has been used in every day clinical practice as a major criteria to select patients for surgical treatment. Nevertheless, a growing body of evidence has been

collected suggesting that it is not only size the of the lesion but also its morphology that is highly related to the thromboembolisation (Fuster, Moreno *et al*. 2005). Development of new clinical markers of plaque destabilization will allow us to perform carotid endarterectomy only on high risk patients, among both symptomatic and asymptomatic lesions. The number needed to treat in order to prevent stroke would be decreased considerably by recognizing vulnerable lesions in small or asymptomatic ones as well as verifying stable plaques among those with severe stenosis. Experimental and morphological studies propose that signs of plaque instability such as inflammation, apoptosis, matrix degradation and neovascularisation predict plaque rupture and consequently thrombotic events. (Loftus, Naylor *et al*. 2000; Morgan, Rerkasem *et al*. 2004; Choudhary, Higgins *et al*. 2006; Higashikata, Yamagishi *et al*. 2006; Tureyen, Vemuganti *et al*. 2006; Ijas, Nuotio *et al*. 2007). In addition, higher lipid content, thinner fibrous cap, more macrophages and plaque rupture have been described in symptomatic lesions (Golledge, Greenhalgh *et al*. 2000). Even though these studies have provided insights into cellular and molecular mechanisms involved in plaque instability, few clinical determinants are used to select patients with vulnerable lesions for intervention apart from the degree of carotid stenosis.

1.2.3.2 Strategies to identify high risk carotid plaques

Given the importance of plaque composition versus degree of stenosis, new strategies to spot vulnerable lesions will include imaging techniques, clinical characteristics and assessment of plaque activity both systemically and locally.

1.2.3.2.1 Biological markers

Today, many of the molecules involved in the inflammatory process of the atherosclerotic lesions can be measured systemically by sensitive assays, and elevated concentrations in the circulation have been shown to be associated with future cardiovascular events. Increased plasma levels of C-reactive protein, monocyte/macrophage colony-stimulating factor and interleukin-6 have been shown to predict a higher cardiovascular event rate (Ikonomidis, Stamatelopoulos *et al*. 2008). Lipoprotein-associated phospholipase A(2) appears to be a specific marker of plaque inflammation that may play a direct role in the formation of a rupture-prone plaque (Weintraub 2008). In CAD patients, the predictive value of monocyte/macrophage colony-stimulating factor was found to be additive and beyond that of C-reactive protein

suggesting the need of a "multimarker approach" in assessing cardiovascular risk. Eldrup and colleagues showed in a prospective study that plasma MMP-9 predicted combined stroke and cardiovascular death, particularly when combined with plaque echolucency (Kietselaer, Reutelingsperger *et al*. 2004).

1.2.3.2.2 Imaging of plaque activity

Activity of a lesion is related to the inflammatory processes, that involves different cell types and a cascade of enzyme-mediated interactions resulting in plaque destabilization. A number of biological relevant molecules such as oxidized LDL, annexin A5 and metalloproteinases have been targeted using scintigraphic imaging. Specific contrast agents for single photon emission computed tomography and positron emission tomography (PET) have been tested (Kietselaer, Reutelingsperger *et al*. 2004; Davies, Rudd *et al*. 2006). The radioisotope 18-fluorodeoxyglucose is taken up by macrophages and these can be visualized to estimate metabolic activity in a plaque (Rudd, Warburton *et al*. 2002).

Contrast-enhanced magnetic resonance imaging is a technique which utilizes molecular contrast based on gadolinium and has a potential of combining gadolinium with small molecules that are involved in physiological processes in a plaque (Winter, Morawski *et al*. 2003). Other methods like photoluminescence and thermography remain to be evaluated and may become additional tools to monitor plaque vulnerability (Casscells, Hathorn *et al*. 1996).

1.2.3.2.3 Invasive imaging of plaque instability

Apart from angiography which has been used to evaluate degree of stenosis and may help in recognition of some morphological features of a lesion (ulceration identified with angiography has a high predictive value for stroke) other imaging techniques such as IVUS and optical coherence tomography have been shown to recognize some characteristics of unstable plaques. Optical coherence tomography is still very much a research tool, while IVUS offers more promise for clinical assessment. IVUS-derived measurements of fibrous cap and lipid core size, appear to correspond closely with histology (Diethrich, Pauliina Margolis *et al*. 2007). However, it is necessary to note that invasive methods are not likely to be used commonly to evaluate asymptomatic carotid lesions due to the risk of thromboembolism (Diethrich, Pauliina Margolis *et al*. 2007).

1.2.3.2.4 Non-invasive assessment of carotid plaque instability

Over the last decade there has been a substantial improvement in different noninvasive imaging modalities that allow characterisation of atherothrombotic plaque structure.

1.2.3.2.4.1 Transcranial Doppler

Physiological tests as transcranial doppler provide information on actual embolic activity of carotid lesions by detecting micro-embolic signals in cerebral artries. This method is also helpful in identifying individuals with cerebral hypoperfusion (Markus, Droste *et al*. 2005; Azarpazhooh and Chambers 2006).

1.2.3.2.4.2 Computer tomography

In many centres, computer tomography (CT) angiography is widely used for preoperative imaging of vascular disorders. Spiral CT is useful to visualize notable morphological characteristics, such as large ulcers. Plaque characterization CT utilizes Hounsfield units to quantify density of the tissue. Oliver and coauthors demonstrated an association between hypodense plaques and necrotic lipid cores, whereas iso-dense plaques corresponded to fibrous ones (Oliver, Lammie *et al*. 1999). Other authors found that spiral CT may achieve the same levels of accuracy as B-mode ultrasound (Wolf, Wehrli *et al*. 2005). Plaque calcification can be optimally quantified with CT. The method appears superior to B-mode ultrasound probably because calcium tends to obscure the view in B-mode ultrasound. The role of calcification in relation to plaque stability remains controversial, with some advocating a protective role, whilst others suggest increased susceptibility to plaque rupture (Wexler, Brundage *et al*. 1996; Nandalur, Baskurt *et al*. 2006).

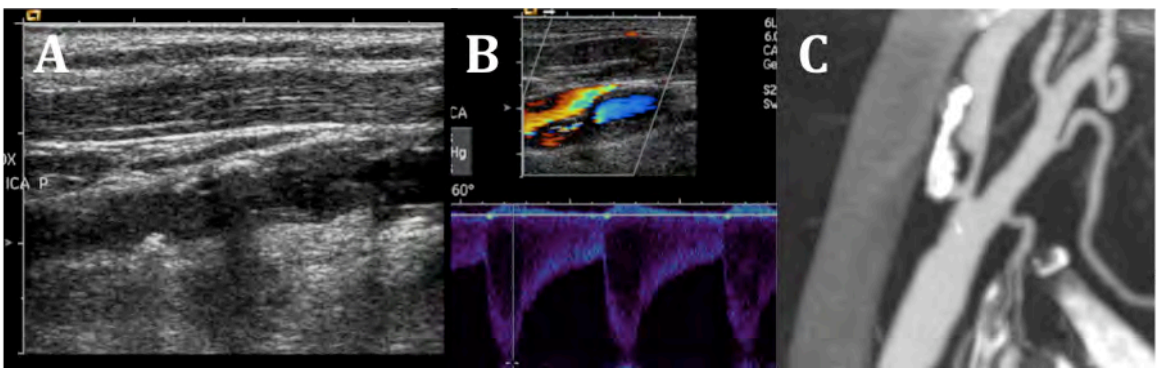


Figure 4. Assessment of carotid stenosis. A – grey scale image; B – Flow assessment with Doppler ultrasound mode; C - Computer tomography angiography

1.2.3.2.4.3 High resolution magnetic resonance imaging

High-resolution magnetic resonance imaging (MRI) has emerged as the potential leading noninvasive *in vivo* imaging modality for atherosclerotic plaque characterisation. At present, some of the major plaque components (fibrous cap, lipid core, intraplaque hemorrhage) can be reliably identified by MRI (Yuan, Mitsumori *et al*. 2001; Trivedi, JM *et al*. 2004). Yuan and coworkers reported on the presence of a ruptured fibrous cap (identified with MRI) in patients who had experienced a stroke or TIA within 90 days (Yuan, Zhang *et al*. 2002). Plaque morphology assessed with MRI does not only correlate with histology but has also been shown to predict late, ipsilateral ischemic events in asymptomatic patients (Takaya, Yuan *et al*. 2006). Takaya and coworkers prospectively studied the association between plaque composition and cerebrovascular events in 154 asymptomatic patients with ultrasonographically proven 50% to 79% carotid artery stenosis over a time period of at least 12 months. They could confirm that thin or ruptured fibrous caps and intraplaque hemorrhage are linked with cerebrovascular events (Yuan, Zhang *et al*. 2002; JM, Tang *et al*. 2008).

Table 1. Assessment of plaque instability

Technique	High risk features	Advantages	Disadvantages
Angiography	Stenosis severity; ulcer	Available in most centres	Only lumen visualized; invasive; stroke risk; contrast related complications; radiation required
CT	Calcification	Rapid, readily available	Contrast related complications; radiation required
MRI (high resolution)	Thin cap, cap rupture, intra-plaque hemorrhage, necrotic core	Non-invasive, able to visualize wall and lumen, less operator dependant than ultrasound	Time required for acquisition; reconstruction and analysis; exclusion of metal implants; availability
Ultrasound	Echolucency, ulcer	Non-invasive, able to visualize wall and lumen.	Operator dependent
TCD	Micro embolic signals	Non-invasive, theoretically a very direct measure of plaque instability	Very labor intensive; complex analysis; prolonged assessment required

Modified from Golledge *et al* (Golledge and Siew 2008)

1.2.3.2.4.4 Ultrasound

Duplex ultrasound technique can be used to study plaque morphology and the degree of echolucency been proposed to predict risk of stroke (Nordestgaard, Gronholdt *et al*. 2003). A great advantage of using Duplex is that the method is broadly available and routinely used to diagnose carotid stenosis and to assess its severity. Both the vessel anatomy, as well as flow can be visualized combining Doppler flow imaging with brightness-mode (B-mode) in duplex ultrasonography. The echogenicity derived from B-mode ultrasound imaging can be either classified subjectively (for example, echolucent, echodense) or in a quantitative manner using gray-scale mean (Gronholdt, Nordestgaard *et al*. 2001). Histologicaly, soft plaques (echolucent) are composed of either lipid, blood or thrombus due to haemorrhage or a combination, are more cellular, while hard plaques (echodense) are more calcified and fibrotic in nature (Goncalves, Moses *et al*. 2003) (Cohen, Tzourio *et al*. 1997). However, ultrasound diagnosis is limited by low soft tissue contrast and low reliability in distinguishing hemorrhage from the lipid core. In the evaluation of plaque inflammation, Gronholdt and colleagues found a strong correlation between echolucent plaques and the amount of macrophages present (Gronholdt, Nordestgaard *et al*. 2002). Other parameters related to an inflammatory phenotype and vulnerable lesion which can be visualized with ultrasound are size of the necrotic core, degree of neoangiogenesis and fibrous cap thickness (Kwee, van Oostenbrugge *et al*. 2008). Grønholdt ML with co-authors were able to show that echolucent plaques, as assessed by B-mode visualisation, carry an increased stroke-risk (Gronholdt, Nordestgaard *et al*. 2001).

A number of studies have demonstrated the possibility to visualize *vasa vasorum* and neovascularisation in the plaques utilizing contrast-based ultrasound technique (Carlier, Kakadiaris *et al*. 2005; Goertz, Frijlink *et al*. 2006).

1.2.3.2.4.5 Diffusion-weighted MR-brain–imaging

Diffusion-weighted MR-brain–imaging (DWI) is highly sensitive to ischemic changes and has been used to show that a large proportion of TIA patients have acute ischemic lesions in the brain (Crisostomo, Garcia *et al*. 2003). Recent studies have suggested that TIA patients with positive DWI are at a high early risk of stroke (Purroy, Montaner *et al*. 2004; Calvet, Touze *et al*. 2009). A meta-analysis also shows that clinical features, which are known to predict a high early risk of stroke after a TIA are also associated

with the presence of acute ischemic lesions on DWI (Redgrave, Coutts *et al*. 2007). DWI has also been used to recognize episodes of silent ischemia during and after carotid artery stenting (Palombo, Faraglia *et al*. 2008; Shibazaki, Iguchi *et al*. 2008). This suggests that some of the patients assumed to have asymptomatic carotid plaques can potentially be converted into a symptomatic group using the DWI technique.

The array of imaging techniques such as DWI, high-resolution MRI and ultrasound have a capability to identify a high-risk subgroup of asymptomatic patients which would benefit from surgical treatment. While these modalities are being further verified, a number of clinical variables are utilized for risk stratification in patients with cerebrovascular disease.

1.2.3.2.5 Clinical parameters

In a recent review, Golledge and Siew pointed out that despite a large number of imaging and biomarker studies, 'presenting symptoms' remains the most clearly identified risk predictor for ischemic stroke in patients with carotid stenosis. At present, no imaging modality or plasma biomarker has clearly identified a high risk sub-group of asymptomatic carotid stenosis for which the benefit of carotid intervention is comparable to that of symptomatic atherosclerosis (Golledge and Siew 2008).

In order to help identify patients at the highest early risk of stroke after a TIA, risk scores (eg, ABCD and California score) have been developed (Johnston, Gress *et al*. 2000; Rothwell, Villagra *et al*. 2000). These scores evaluate risk by taking into account age, blood pressure, diabetes, and the nature of ischemic symptoms. The ABCD score, which was derived from the ECST data, effectively stratifies the short-term risk of stroke following TIA into those with a high (12%), moderate (6%), and low (1%) 7-day stroke risk (Redgrave, Coutts *et al*. 2007). These models are useful to identify subjects with a high probability of a new embolic event a short time after the index symptom, but do not help in risk stratification for other groups of patients.

Indeed, patients with asymptomatic carotid lesions have an annual stroke risk of approximately 2% compared with 5% for symptomatic patients within the first two weeks after the index event (Giles and Rothwell 2007). The type of ischemic symptom is another factor that affects the probability of new embolisation and patients with TIA or minor stroke have a higher risk than patients with amaurosis fugax (Rothwell, Eliasziw *et al*. 2004). An important fact is that the risk of recurrent embolization is most pronounced within the first two weeks after the index event and plaque morphology changes with time after stroke and TIA (Coull, Lovett *et al*. 2004; Kleindorfer, Hill *et al*.

2005; Rothwell 2008). It was demonstrated on data from the ECST and NASCET that the randomisation within 2 weeks after the last ischemic event increased the effectiveness of surgery ($p = 0.009$) (Rothwell, Eliasziw *et al*. 2004). The number of patients needed to treat to prevent one ipsilateral stroke in 5 years was five, for those randomised within 2 weeks after the last symptom compared with 125 for those randomised after more than 12 weeks.

These clinical features may represent plaque rupture followed by a healing response in the ruptured plaque involving repair mechanisms with SMC proliferation and synthesis of extracellular matrix components (Burke, Kolodgie *et al*. 2001).In the respect, histological findings that plaque inflammation decreased with time after a stroke but persisted after a TIA suggests different healing patterns in the lesions (Redgrave, Lovett *et al*. 2006).

1.2.3.3 Assessment of healing responses in human carotid plaques

Taking into account that the repair processes after plaque rupture may be reflected by clinical outcomes and morphological characteristics, it is feasible to use these parameters to assess healing activity and study the biology of this process in human samples. One of the available tools is the gene array technique, which provides information on mRNA expression of thousands of single genes simultaneously. By applying this system on atherosclerotic lesions obtained from carotid endarterectomies, new molecular mechanisms and cellular processes in plaque stabilization and rupture can be studied. At the same time, new imaging techniques should be advanced further so that known morphological features of plaque rupture and repair can be visualized in every day clinical practice for risk stratification. These methods could be used for investigation of regulatory mechanisms of plaque disruption and healing in combination with gene and protein assays.

1.2.3.4 Surgical treatment of atherosclerosis

Surgical treatment of atherosclerosis is used for prevention of thromboembolism or improvement of organ perfusion compromised by arterial disease. By performing a thromboendarterctomy (TEA), a source of potential plaque rupture and thrombosis is removed from an artery. TEA is the common procedure used for treatment of carotid stenosis and the iliofemoral segment of lower limb arteries (Crawford, Chung *et al*. 2007; Antoniou, Koutsias *et al*. 2008). By-pass surgery is performed in order to improve

blood supply distally to a lesion. Aortocoronary bypass remains a common technique for patients with multiple and prolonged coronary lesions (Taggart 2009). In treatment of PAD, bypass procedures are also used, especially for treatment of distal and long occlusive lesions (Norgren, Hiatt *et al*. 2007). Autologous veins serve as grafts when possible, otherwise synthetic grafts may be utilized. Some atherosclerotic lesions can be treated with an endovascular approach – when surgical exposure of the vessel is not needed and the plaque is reached through the lumen via fluoroscopy.

This technique is widespread and provides a less traumatic treatment for a significant portion of patients with CAD and PAD. Usage of endovascular treatment of carotid artery stenosis is limited and the rationale of this method is being tested in a number of trials (Hart 2008; Ederle, Featherstone *et al*. 2009). The most common type of endovascular treatment is angioplasty, dilatation of a stenosed segment with a balloon catheter inserted through the lumen. This allows for reopening of the vessel without removing the plaque. In order to prevent elastic recoil, - a phenomenon when the dilated segment returns to its original shape after angioplasty, a metal scaffold structure, a stent, is often placed inside the artery. The field of endovascular surgery is growing and progressing at a high pace and new better devices are constantly being tested and launched commercially. With special instruments, plaque material can also be partly removed from the vessel resulting in an "endovascular endarterectomy" which is normally combined with stenting.

An angioplasty can also be done subintimally (Met, Van Lienden *et al*. 2008). With this technique, a guidewire followed by an angioplasty balloon are introduced into the subintimal space, creating a new lumen between the intima and media. Sent deploymet

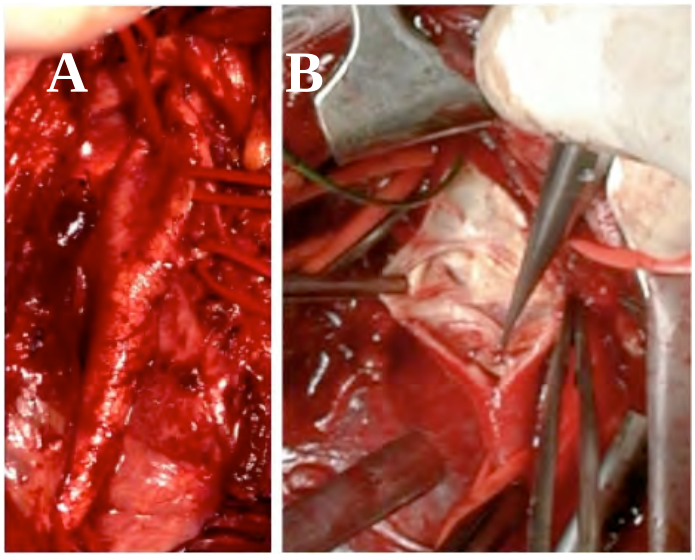


Figure 5. Carotid endarterectomy. A- carotid bifurcation. B- Artery opened to remove the plaque

is often necessary after this procedure. In contrast to conventional angioplasty, this method does not injure the surface of the lesion and may thus be associated with less thromboembolism.

Another method which does not require extensive dissection but at the same time provides a possibility to perform TEA on long segments, is ringstripping. With a special ring-formed instruments a regular TEA can be prolonged over unexposed parts of the vessel (Antoniou, Koutsias *et al*. 2008).

1.2.4 Restenosis

Patency of a successful revacularisation can be hampered by restenosis, a recurrent narrowing at the site of initial intervention for an atheromatous stenosis. In contrast to de novo atherosclerotic lesions, restenosis develops relatively quickly after surgery, typically between 1 to 6 months, up to 1 year. A 50 % constriction of the operated segment is called restenosis, but a comparatively minor portion of these lesions cause clinical (symptomatic) restenosis and recurrence of ischemia (Cutlip, Chauhan *et al*. 2002). Clinical restenosis often requires repeat revascularization procedures.

The phenomenon of restenosis became of a great importance when percutanious coronary angioplasty (PCA) developed into a standard procedure for treatment of CAD with restenosis rates of about 30 % - 50 % after angioplasty. The three main components of restenosis that have been described are: elastic recoil, negative remodeling of the treated vessel and intimal proliferation. Elastic recoil is a re-contraction of the dilated vessel wall which occurs immediately and the vessel shrinks back. Negative remodeling refers to the gradual reduction of the vessel diameter during days to months after balloon dilation (Schwartz 1998). In both recoil and remodelling, the medial layer shrinks and becomes fibrotic. Both recoil and remodeling can be prevented by the implantation of a stent. Stent placement decreases restenosis rates from about 25-30% to 10-15% after PCA and from 45% to 25 % after angioplasty of the femoral artery (Schillinger, Sabeti *et al*. 2007). The radial force of contemporary stents is sufficient to hinder practically all shrinking of the vessel. As a result, the only restenosis process which compromises results of percutaneous transluminal angioplasty (PTA) with stenting is intimal hyperplasia. Stent struts penetrate the artery wall causing a continuous inflammatory response and cellular proliferation (Orford, Selwyn *et al*. 2000). Development of stents which are covered with a polymer slowly releasing antiprolifrative agents, exerting a local effect on the vessel wall, became a true step forward in the prevention of restenosis

decreasing its rate down to 3-5% (Holmes, Leon *et al*. 2004) (Stone, Ellis *et al*. 2005). During the last 5-7 years, drug eluting stents (DES) have been increasingly used to treat CAD and some publications support the use of DES in noncoronary vascular beds (Bosiers, Cagiannos *et al*. 2008). Though DES dramatically improved results of PCA, this approach does not make restenosis an irrelevant problem for interventionists or vascular surgeons. Both experimental and clinical data have recently indicated that DES can cause delay reendothelisation and cause late thrombosis and hence, myocardial infarction (Virmani, Farb *et al*. 2004; Pfisterer, Brunner-La Rocca *et al*. 2006; Stahli, Camici *et al*. 2009). Secondly, a group of patients who tend to develop restenosis despite treatment with DES (individuals with diabetes and complex lesions) is growing and more and more of these patients will be treated with an endovascular approach (Dibra, Kastrati *et al*. 2005). The use of a stent for treatment of distal lesions in the peripheral vascular bed is limited (Norgren, Hiatt *et al*. 2007). Importantly, open vascular interventions are also associated with recurrent lumen loss due to restenosis, though at lower rates than endovascular procedures (Beard 2008).

Incidence of restenosis after carotid TEA varies due to the differences in the definition of restenosis and is approximately 6–14% (Lattimer and Burnand 1997). Frericks H with coauthors calculated risk of recurrent stenosis of 10% in the first year after surgery (Frericks, Kievit *et al*. 1998). Intimal hyperplasia takes place also after bypass procedures and limits outcome of both coronary and peripheral revascularisation. Occlusion of vein grafts is observed in approximately 15–25% of patients during the first postoperative year and in over 50% after 10 years (Murphy and Angelini 2004; Hata, Sezai *et al*. 2007). Intimal hyperplasia develops at the site of anastomosis and over the length of the vein, this process is also called vein graft disease. Both the artery and the vein are exposed to injury during the intervention in a similar manner as in PTA. However, injury of a vein graft also consists of mechanical and hemodynamic consequences to a vein due to handling during harvesting, ischemia/reperfusion, and sudden exposure to the arterial circulation. The structural and physiological differences between vein and artery suggest also differences in the healing response to injury (LoGerfo, Quist *et al*. 1983) (Hinokiyama, Valen *et al*. 2006; Owens, Wake *et al*. 2006).

1.2.4.1 Intimal hyperplasia

The healing reaction of the vessel wall after injury has been widely studied during the past decades and the understanding of cellular and molecular mechanisms of intimal hyperplasia has been gained mainly from animal models. A similar response to a diverse

repertoire of injuries suggests an unspecific and uniform repair mechanism behind intimal hyperplasia. Disruption of endothelium leads to adhesion of platelets to the exposed subendothelial ECM. Activated platelets release a large variety of cytokines and growth factors, which initiate SMC proliferation, leukocyte recruitment, and activation of the coagulation cascade. Injured endothelial cells (EC) and SMCs can also be a source of cytokines and the maximal intimal response requires damage of medial SMCs (Fingerle, Au *et al.* 1990). Platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-β, interleukin (IL)-1, IL-6, IL-8, thrombin, adenosine diphosphate, and thromboxane A2 are some of the substances which are released and activated after injury. A portion of the surrounding SMCs will also undergo apoptosis early after injury (Beohar, Flaherty *et al*. 2004; Clarke, Figg *et al.* 2006). SMCs, which are normally quiescent, undergo a process of dedifferentiation and change their phenotype from a contractile to a synthetic state (Thyberg, Blomgren *et al.* 1995). Within the first days, medial SMCs begin to proliferate under stimulation of FGF-2 (Lindner, Lappi *et al.* 1991). Other factors such as insulin-like growth factor (IGF)-1, thrombin , TGF- β, together with cytokines IL-1β and IL-6, all contribute to SMC proliferation (Hayry and Yilmaz 1995);(Zhu, Zhao *et al*. 2001) (Gallo, Padurean *et al*. 1998) (Wolf, Rasmussen *et al*. 1994; Rectenwald, Moldawer *et al*. 2000). After a few days, proliferation of SMCs in the media reaches its peak and SMCs begin to migrate to the intima. Migration requires degradation of basement membrane which surrounds medial SMCs (Hedin, Roy *et al*. 1999). Furthermore chemotactic factors such as PDGF and IGF-1 are involved in migration (Jackson, Raines *et al*. 1993; Englesbe, Davies *et al*. 2004). At this stage, growth factors are not only released by platelets but also from storage in the ECM and are synthesized by SMC in an autocrine and pararine manner (Majesky, Schwartz *et al*. 1987).

In addition to SMCs derived the from media, it has been shown that adventitial myofibroblasts and circulating progenitor cells can reconstitute the SMCs in the noeintima (Zalewski, Shi *et al*. 2002; Yokote, Take *et al*. 2003).

In rodent models of intimal hyperplasia SMC proliferate for up to 2 weeks and begin to synthesize extracellular matrix components, such as elastin, collagen, glycoproteins, and proteoglycans forming neointimal layer. The neointima reaches a steady state between 1 and 3 months, the time period which is required for reendothelisation (Nikkari, Jarvelainen *et al*. 1994).

1.2.4.2 Inhibition of intimal hyperplasia

Many growth factors and cytokines has been shown to regulate function of SMC after injury (Lindner, Lappi *et al*. 1991; Wolf, Rasmussen *et al*. 1994; Hayry, Myllarniemi *et al*. 1995; Gallo, Padurean *et al*. 1998; Zhu, Zhao *et al*. 2001);(Rectenwald, Moldawer *et al*. 2000). Some of these molecules and the downstream signaling pathways have been targeted to prevent and attenuate intimal hyperplasia, such as antibodies against integrin alphaVbeta3, PDGF and FGF-2; anti C-myc and C-myb antisense oligonucleotides and a number of other agents (Davies and Hagen 1994). The approach to specifically target one mediator often showed some effect *in vitro* and in animal studies but, while adding to understanding of the pathobiology of the disease, most have failed to cross the boundary between experimental models and the clinical situation (Davies and Hagen 1994). Less specific therapies such as antiplatelet agents, anticoagulants or antioxidants had a similar outcome. However even less specific approach became a success when antiproliferative drugs (rapamycin or paclitaxel) were used. Prevention of coronary restenosis became possible when these agents were delivered locally to the site the angioplasty on a drug eluting stent (Holmes, Leon *et al*. 2004; Stone, Ellis *et al*. 2005). This changed clinical practice for the treatment of CAD (Virmani, Farb *et al*. 2004; Pfisterer, Brunner-La Rocca *et al*. 2006; Stahli, Camici *et al*. 2009).

At the present when new pathways involved in intimal hyperplasia have been investigated and the technology for local drug delivery has been developed, it may be time to reconsider specific targeting of growth factors signaling with new compounds and cutting edge delivery systems.

1.2.5 Role of the the IGF-1 system in vascularature

1.2.5.1.1 IGF1 is important for cell proliferation, differentiation, transformation, and survival

Insulin-like growth factor 1 (IGF-1) is a small peptide hormone with molecular weight 7649 daltons and is one of the main mediators of the actions of growth hormone. IGF-I is probably the most potent anticatabolic and anabolic hormone in humans (Jones and Clemmons 1995). This peptide is mainly synthesized by the liver and has endocrine effects in a number of target tissues. A family of at least six IGF binding proteins (IGFBPs), regulate the bioavailability of IGF-1. IGFBPs have a high affinity for IGF-1 and most circulating IGF-1 molecules are held as a complex with IGFBPs. Postnatally, its long-term function may lie primarily in carbohydrate and lipid homeostasis as well as

healing response to injury when IGF-I can be secreted by almost all cell types in an autocrine and paracrine fashion (Sjogren, Liu *et al*. 1999). The type 1 IGF receptor (IGF-1R) is a heterotetramer composed of two subunits that contain the hormone binding domain, which are linked to two subunits that contain tyrosine kinase catalytic activity domains by disulphide bonds (Riedemann and Macaulay 2006). Upon ligand occupancy the receptor undergoes a conformational change that activates the tyrosine kinase activity, which then activates downstream signaling molecules by protein phosphorylation. Stimulation of the IGF1 receptor initiates signaling pathways involved in cell proliferation, differentiation, transformation, and survival. IGF1R-dependent signaling is crucial for the survival of many cell types including vascular SMCs (Delafontaine, Song *et al*. 2004). A major downstream effector of IGF1R signaling involves autophosphorylation and subsequent tyrosine phosphorylation of Shc and insulin receptor substrate (IRS) -1, -2, -3, and -4. IRS serves as a docking protein and can activate multiple signaling pathways, including phosphatidyl inositol 3-kinase (PI3K), Akt, and mitogen-activated protein kinase (MAPK) (LeRoith, Werner *et al*. 1995; Saltiel and Kahn 2001; Tsuruzoe, Emkey *et al*. 2001). Akt phosphorylates a large number of targets involved in glucose metabolism and cell differentiation, proliferation, and survival (Stewart and Rotwein 1996; Allard, Figg *et al*. 2008). A number of articles suggests an important crosstalk between other growth factors that are involved in repair processes, such as PDGF, FGF, epidermal growth factor and IGF1 (Arnqvist, Bornfeldt *et al*. 1995; Swantek and Baserga 1999; Lassarre and Ricort 2003).

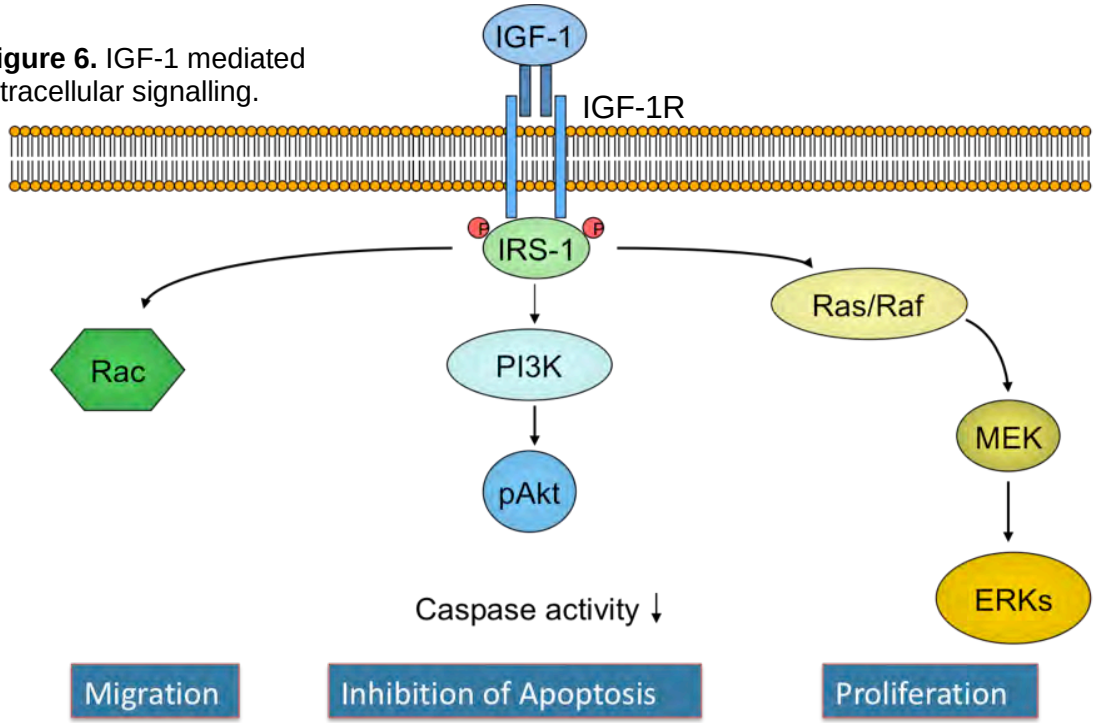


Figure 6. IGF-1 mediated intracellular signalling.

1.2.5.1.2 The IGF-1 system and vascular SMCs

IGF-1 has been shown to have mitogenic, antiapoptotic, and promigratory effects on vascular SMCs (Delafontaine, Song *et al*. 2004). It binds to IGF-1R expressed by vascular SMCs. A number of studies suggest that IGF-1 plays an important role in proliferation, migration, and inhibition of apoptosis in SMCs during restenosis development (Arnqvist, Bornfeldt *et al*. 1995). Increased levels of IGF-1 and IGF-1R messenger RNA were found in *de novo* and in restenotic coronary lesions compared with normal coronary arteries (Grant, Wargovich *et al*. 1996). Overexpression of IGF-1 in transgenic mice led to the development of intimal hyperplasia (Zhu, Zhao *et al*. 2001). Thrombin, PDGF, bFGF, angiotensin II, and estrogen have been shown to influence the levels of IGF-1 and IGF-1R mRNA in vascular SMCs (Delafontaine, Song *et al*. 2004).

1.2.5.1.3 Role of the IGF-1 system in atherosclerosis

There is a evidence that the IGF-1 system has a protective role in the cardiovascular system. Low levels of IGF-I and its binding proteins have been associated with cardiovascular disease, suggesting that both lowered IGF-I and IGFBP-1 levels increase cardiovascular risk (Ezzat, Duncan *et al*. 2008). IGF-1 can stimulate production of nitric oxide (NO) from ECs and SMCs, which may have beneficial effects on the vascular system (Walsh, Barazi *et al*. 1996). In advanced atherosclerotic plaques levels of IGF-1 and IGFR-1 were found decreased what related to high apoptosis rates (Okura, Brink *et al*. 2001; Patel, Zhang *et al*. 2001)). Allard D with coauthors also reported that SMCs derived from plaques were prone to apoptosis and exhibited a defect in IGF1-dependent survival signaling (Allard, Figg *et al*. 2008). The same authors found that oxidative stress reduced IGF1R expression and induced SMC apoptosis in culture.

Indirect attenuation of IGF-1 signaling has on the other hand been shown to cause decrease in atherosclerotic lesion size and progression in pigs (Nichols, du Laney *et al*. 1999). IGF-1 can also potentiate platelet aggregation, via the IGF receptor/PI3K/PKB pathway (Hers 2007). Nevertheless in a recent study treatment with recombinant IGF-1 reduced size of atherosclerotic lesions in ApoE knock out mice (Sukhanov, Higashi *et al*. 2007).

SMC proliferation and migration contribute to healing processes in atherosclerotic plaques and the IGF-1 system may thus be important for plaque stabilization. At the

same time, the mitogenic activity of IGF-1 can promote development of intimal hyperplasia and restenosis. It is important to stress that IGF-1 can be crucial for neovessel formation in atherosclerotic lesion and may therefore influence intraplaque hemorrhage, a critical event in plaque instability. A therapy which would decrease intraplaque neovascularisation could alleviate the risk of rupture by slowing the rate of necrotic core expansion by eliminating accumulation of RBC-derived cholesterol (Kolodgie, Narula *et al*. 2007).

1.2.5.1.4 Targeting IGF-1 to inhibit intimal hyperplaia

The IGF-1 system has been targeted in animal models of intimal hyperplasia, using both pharmacotherapy and genetic approaches to prevent SMC proliferation. Angiopeptin and octreotide are two somatostatin analogs that down-regulate the production of growth hormone and have therefore been used to block the growth hormone/IGF-1 axis to prevent neointima formation (Yumi, Fagin *et al*. 1997). But in clinical trials neither octreotide or angiopeptin could show any beneficial effect on restenosis (Eriksen, Amtorp *et al*. 1995; von Essen, Ostermaier *et al*. 1997). Hayry *et al* reported decreased SMC proliferation with an inhibitory stable D-peptide analogue of IGF-1, but without significant inhibition of neointima formation in the rat carotid artery balloon injury model (Hayry, Myllarniemi *et al*. 1995). Truncation of the carboxyl terminal end of IGF-1R has also been shown to suppress neointimal formation (Lim, Park *et al*. 2004).
Together these data imply that interference with the IGF-1 axis in SMCs has a potential to inhibit development of intimal hyperplasia. So far, however, no clinical studies using drugs that affect the IGF-1/IGF-1R pathway have shown any benefit in the prevention of restenosis (Eriksen, Amtorp *et al*. 1995; von Essen, Ostermaier *et al*. 1997). In parallel, IGF-1 and IGF-1R have been objects of interest in the field of cancer research. IGF-1 is a generalized systemic growth factor and because IGF-1R is expressed in many tumour cells IGF-1 is believed to play a critical role in the development and progression of human cancer (LeRoith and Roberts 2003). Recent studies demonstrated that the IGF-1 receptor can be an important target for cancer therapy since the receptor is crucial for the survival and growth of most types of cancer cells. The high structural similarity between IGFR-1 and the almost identical insulin receptor, which is critical for metabolism of normal cells, has been a major obstacle in finding molecules that selectively inhibit the IGF-1 receptor. Most IGF-1 receptor inhibitors currently being developed are systemically administered monoclonal antibodies (Clemmons 2007). A group from Karolinska Institutet found that the cyclolignan picropodophyllin (PPP) can specifically

inhibit IGF-1R phosphorylation without affecting the insulin receptor (Girnita, Girnita *et al*. 2004). The inhibitory effects of PPP on IGF-1R phosphorylation and on malignant cell growth have been studied both *in vitro* and *in vivo*, and PPP treatment has been shown to cause tumor regression in several mouse cancer models (Girnita, All-Ericsson *et al*. 2006; Menu, Jernberg-Wiklund *et al*. 2006). Furthermore Vasilcanu *et al* recently reported that PPP can downregulate the cellular expression of the IGF-1R (Vasilcanu, Vasilcanu *et al*. 2008). These findings suggest that PPP may be also used to prevent IGF-1R activation in vascular SMCs and affect neointimal formation after vascular injury.

Taking in to account the important role which IGF-1 system plays in diseased or injured vessel wall, the possible effect of PPP treatment on atherosclerosis progression should be evaluated.

1.2.6 Effects of fluid shear stress on EC and SMC physiology

Local hemodynamic factors have substantial effects on both structure and function of the vessel wall and ECs together with SMC react on hemodynamic changes in order to maintain blood flow. Fluid shear stress, the frictional force per unit area from flowing blood, acts mainly on the endothelial cells that line the vessels. Blood pressure, which drives fluid flow, causes circumferential stretching sensed by the cells in all layers of the wall. Elevated pressure triggers SMCs contraction, which narrows small resistance arteries to keep blood flow constant in downstream capillaries. If pressure remains elevated over longer periods, SMCs engage in remodeling, which thicken the vascular wall to resist these forces. This results in endothelial, SMC, and fibroblast hyperplasia and hypertrophy in a manner that compensates for the increase in pressure to retain the original lumen size. At the same time vessel wall diameter will change in response to local shear stress alterations in order to preserve optimal lumen dimensions.

On healthy subjects it has been shown that carotid intima-media thickening (IMT) is negatively correlated to carotid shear stress (Carallo, Irace *et al*. 1999). In the case of atherosclerotic human coronary arteries, at least two processes have been described to maintain a normal lumen size. When shear stress is high (>38 dyne/cm^2), arteries remodeled by decreasing plaque area and increasing lumen without changes in vessel size. At sites with low shear stress (<9 dyne/cm^2), lumen was maintained by an increase in the outer vessel size. Both processes occurred at intermediate values of shear stress (9-38 dyne/cm^2) (Stone, Coskun *et al*. 2003).

For EC flow is a potent survival signal and it has effects on multiple signaling pathways, which include the PI3K, mitogen-activated protein kinase 7 (MAPK7) and NO pathways (Li, Haga *et al.* 2005). In some regions blood flow is laminar and has same direction during the cardiac cycle. In regions where arteries divide or curve sharply, there are regions in which complex flow patterns develop. Flow in these regions is reduced and can reverse direction during the cardiac cycle — so-called oscillatory flow. EC in regions of laminar shear have a quiescent, anti-inflammatory phenotype: alignment in the direction of flow, expression of anti-inflammatory genes and low levels of oxidative stress, cell turnover and permeability. These regions are protected from atherosclerosis. In contrast, ECs in regions of disturbed flow display an activated, pro-inflammatory phenotype that is characterized by poor alignment, high turnover, oxidative stress, expression of inflammatory cytokines (Hahn and Schwartz 2009). These sites are more prone to development of atherosclerosis than areas with an undisturbed, laminar shear stress (Yoshizumi, Abe *et al.* 2003).

It is mainly ECs which directly sense shear stress although SMC can also be exposed to fluid shear stress (FSS) even under physiological conditions. Tada *et al* showed that flow can affect SMCs through fenestral pores in internal elastic lamina (Tada and Tarbell 2002). Furthermore, after various interventional procedures to the vessel wall, the endothelial layer may be denuded exposing SMC for the blood flow. In the rat carotid artery low blood flow after balloon injury promotes a decreases in lumen size due to inward remodeling (Ward, Tsao *et al.* 2001). In addition, high blood flow inhibits intimal hyperplasia after arterial injury (Kohler and Jawien 1992; Qin, Dardik *et al.* 2001). Activated medial SMCs migrate towards the lumen and form neointima. These cells remain exposed to the flow directly until reendothelisation is accomplished. FSS has been shown to suppress proliferation of SMCs and induce apoptosis *in vitro* (Sterpetti, Cucina *et al.* 1993; Fitzgerald, Shepherd *et al.* 2008). Importantly, not only arterial but also the venous wall can be exposed to abnormal flow conditions. Particularly after bypass procedures or creation of arterio-venous fistulas. Arterial flow has an impact on the venous wall which is an additional injury to the mechanical damage and often lead to loss of ECs. Up to now, it is mainly effects of shear stress on ECs that have been studied, but further investigation of mechanisms by which SMC sense and react on flow conditions will be of great value in order to develop strategies for prevention of restenosis and treatment of atherosclerosis. This requires new physiological models that can be subjected to genomic and proteomic analyses, because

flow-dependent vascular remodeling involves multiple cell types and molecular processes.

1.3 VESSEL WALL HEALING IN ANIMAL MODELS

In 1714, Stephen Hales opened an artery of a horse, inserted a brass tube, and measured the pressure of the blood. This was an accurate experiment demonstrating that the heart exerts pressure in order to pump blood; however, another result of the experiment was the horse's death.

Animals have been used to study biology and physiology for many centuries. With growing understanding of vascular biology and cardiovascular disorders, animal models of atherosclerosis, thrombosis, intimal hyperplasia and restenosis, arteritis and aneurysmal disease were developed (Moghadasian 2002; Touchard and Schwartz 2006; Whinna 2008) (Luzina and Handwerger 2000; Chaer, DeRubertis *et al*. 2006). Though animal models are often criticised for not perfectly reflecting all features of a complex disease, they allow to scrutinize certain pathological mechanisms. For example, the rat carotid injury model has been used to study SMC behaviour *in vivo*. It is also possible to reproduce more complex conditions, study vascular physiology, assess the role of environmental factors, and evaluate new drugs.

Since healing responses take place in the vessel wall under different situations, such as endothelial injury, atherosclerosis or restenosis, various models are required to study this process.

1.3.1 Animal models of atherosclerosis

Until the early nineties, diet-induced atherosclerosis models were commonly used, but the lesions tended to be limited and did not reflect all stages of human disease. These models were also criticized because of the toxicity and inflammatory responses due to the diet. In 1992 the first line of gene targeted animal models, namely apolipoprotein E -

/- knockout mice was developed and this represented a paradigmatic shift in of atherosclerosis research. Of the genetically engineered models, the apoE -/- deficient model is the only one that develops extensive atherosclerotic lesions on a chow diet. It is also the model in which lesions have been characterized most thoroughly. The lesions develop into fibrous plaques in the aortic root, aorta and large arteries. The main disadvantage of this, and other atherosclerosis mouse models, is that plaque rupture with thrombosis and end organ ischemia do not occur thus limiting comparisons with a critical component of human atherosclerosis. Whether plaque erosion and disruption of the fibrous cap do take place is a matter of controversy and further research will elucidate this fact (Jackson, Bennett *et al*. 2007; Schwartz, Galis *et al*. 2007). Seo at al recently reported that Apo E -/- mice had signs of plaque disruption with hemorrhage (Seo, Lombardi *et al*. 1997). Similar lesions were later reported by Renard *et al* in the LDL receptor -/- mouse with atherosclerosis accelerated by diabetes and at a lower frequency in apoE -/- mice used as a control (Renard, Kramer *et al*. 2004; Gough, Gomez *et al*. 2006).

1.3.2 Animal models of intimal hyperplasia

A number of different species have been used to model intimal hyperplasia: rodents (including rats, mice, rabbits), pigs, dogs, or primates. Because intimal hyperplasia is to a large extent an unspecific reaction, diverse types of injury methods are used in these models including mechanical injury (overstretch artery with noncompliant angioplasty balloons inflated to high pressures, very compliant, low-pressure balloons for denudation injury or directional atherectomy) or injury induced by agents such ethanol, air desiccation, irradiation or inducing severe inflammation with copper stents by foreign body implants (Touchard and Schwartz 2006). Many authors found it important to try to reproduce interventions in humans by either placing animals on a high-fat diet or with complementary injuries. All types of arterial injury begin with endothelial denudation, and progress to deeper injury. In the rat carotid model, injury typically includes endothelial denudation, without disruption of the internal elastic lamina (IEL) or major media damage. In this model the endothelium is stripped uniformly from the common carotid artery by passing an inflated Fogarty balloon catheter several times through the artery over its length from the aortic arch to the bifurcation (Clowes, Reidy *et al*. 1983). This mild injury contrasts to deeper arterial injury usually described in the rabbit iliac and porcine coronary arteries, when IEL and medial dissection is similar to

the damage following human PTA. In human restenosis an inflammatory reaction takes place after injury, that leads to the activation of SMC, recruitment of monocytes and progenitor cells. At the same time, there is remarkably little inflammatory response to injury in the rat carotid model at least within the intima and the media. In contrast hypercholesterolemic rabbits, porcine and nonhuman primate models show robust inflammatory reactions to injury with mononuclear cell infiltration (Schwartz, Holmes *et al*. 1992; Miyauchi, Aikawa *et al*. 1998). Rat studies initiated the concept of SMC proliferation and migration from media to intima, which is a common feature of most of other models. Cell proliferation and migration in rats, mice and pigs begins early after denudation (1 or 2 days) and proceeds for 2 to 4 weeks (Zempo, Koyama *et al*. 1996). The rabbit iliac model shows proliferation over the same period with peak at 1 week (Stadius, Gown *et al*. 1994).

Since the molecular biology of smooth muscle cell proliferation is best described in the rat carotid artery model, new treatment strategies could be screened in the rat before testing in other more advanced restenosis models.

It is possible to mimic surgical intervention on small animals and therefore approximate responses of the vessel wall to that in patients. Injury procedures similar to endarterectomy can be performed on rodents or pigs, as well as bypass creation using vein or synthetic materials. Organ transplantation can be modeled by transplanting a vein or arterial graft from another animal or species. Balloon angioplasty with stenting is widely performed on porcine coronary arteries which have similar anatomical and physiological characteristics with human coronaries (Schwartz, Edwards *et al*. 1994), but can also be tested in rabbits and rats.

In the various animal models, molecular and cellular mechanisms of vascular repair can be studied, as well as the impact of new therapeutic strategies or the role of risk factors. One of the benefits of working with animals is the access to tissues, which can be harvested at various time points and analysed using histology gene expression and protein assays. Another alternative is to use *in vivo* imaging methods to evaluate changes in vascular morphology in live animals.

1.3.3 Imaging in-vivo

The obvious advantage of non-invasive *in vivo* imaging over conventional microscopy techniques is that the latter involves chemical fixation of removed tissues from which it

can be difficult to generate functional data (Phair and Misteli 2001). Furthermore, longitudinal imaging of the same animal model at multiple time points using bio-imaging assays can generate more valuable information than would be obtained from multiple individual animals. In this case, the animal acts as its own control and dynamic data can be collected without the sacrifice of a large number of animals (McVeigh 2006).

Another important application of *in vivo* imaging studies on animals is the development of new visualization techniques. Recent advances in visualization technology allow us to utilize the same modalities for clinical and experimental studies, and use of the same parameters and end points for animals and humans.

There has been considerable progress in the development of non-invasive small animal *in vivo* imaging technology. Magnetic Resonance Imaging, Computer Tomography, Positron Emission Tomography and optical imaging (bioluminescence and fluorescence) are the most popular techniques utilised by researchers over recent years.

Magnetic resonance imaging has been effectively used for visualization of vessels in both rat and mouse(Weinreb, Aguinaldo *et al*. 2007). There is considerable evidence that MRI provides a precise assessment of changes in vessel wall dimensions associated with lesions throughout the aorta in ApoE -/- mice (McAteer, Schneider *et al*. 2004; Schneider, McAteer *et al*. 2004). This technique has also been tested for both cellular and molecular imaging *in vivo* in order to track changes of plaque structure relevant to progression, stabilization, rupture and healing of plaques (Amirbekian, Lipinski *et al*. 2007; Hyafil, Cornily *et al*. 2007).

Langheinrich AC *et al* developed a method to visualize *vasa vasorum* of aortas from apoE–/–/LDL–/– mice with micro-computed tomography. They could also characterize plaque volume and CT "density" as well as *vasa vasorum* luminal volume along the aorta. Moreover, they showed that adventitial vasa vasorum communicate with intraplaque microvessels (Langheinrich, Michniewicz *et al*. 2006). This approach required sacrifice of animals but provided unique three dimentional reconstruction of lesions, vasa vasorum and intraplaque vessels. Phase-contrast X-ray CT imaging has great potential to reveal structures inside biological soft tissues, because its sensitivity to light elements is almost 1,000 times greater than that of absorption-contrast X-ray imaging. Even intraplaque haemorrhage could be detected with dual-energy CT imaging of iron deposits in genetically modified mice (Langheinrich, Michniewicz *et al*. 2007).

PET has high sensitivity and permits accurate quantification of molecular targets. Nahrendorf with coauthors reported a possibility to directly detect macrophages in

atherosclerotic plaques in mice. They achieved improved sensitivity, ability to readily quantify the PET signal and perform whole-body vascular visualisation (Nahrendorf, Zhang *et al*. 2008).

As well as in humans, ultrasound visualisation techniques are widely spread in animal research. Especially in the field of vascular biology, where ultrasound (US) offers a unique combination of morphological imaging and flow assessment with Doppler function. B-mode ultrasound generates images based on interactions between tissues and sound waves generated by the mechanical displacement of small piezoelectric crystals in the transducer. Acoustic echoes are generated at boundary zones between different tissues, or tissue constituents by ultrasound scattering.

However, due to the limitations of spatial resolution with a conventional clinical US system designed for human use, the image acquired was hardly adequate for accurately quantifying lumen and vessel wall dimensions in mice and rat. A system with significantly higher frequency of the US signal (50 MHz compared with up to 15 MHz in clinical applications) and therefore notably better spatial resolution (up to 30 μm) has been recently developed by VisualSonics™ (Toronto, ONT, Canada). Their Vevo technology is applicable for accurate quantification of vascular dimension and wall thickness in rat and mice. The US visualization with very high spatial resolution, also called ultrasound bioicroscopy (UBM), was applied to different rodent models of cardiovascular pathology. The possibility to follow the natural development of plaques in ApoE knockout mice has been described by Gan *et al* (Gan, Gronros *et al*. 2007). Martin-McNulty *et al* applied UBM to study aneurysm development in a mouse model (Martin-McNulty, Vincelette *et al*. 2005). Goldberg *et al* demonstrated that aneurysm morphology can be quantified using 3-D reconstructions of US images in a mouse model of accelerated aneurysm formation(Goldberg, Pakkiri *et al*. 2007). UBM with contrast microbubbles targeted to vascular endothelial growth factor receptor 2 was tested for noninvasive visualization receptor expression in tumor vessels in mice (Willmann, Paulmurugan *et al*. 2008). This approach may be of a great value for noninvasive *in vivo* assessment of vasa vasorum and plaque neovascularisation in rodents.

Intravascular ultrasound has also been applied for small animal models and Rochefort with coauthors were able to measure vessel wall thickness of the rat carotid artery with 40 MHz IVUS probe (Rochefort, Mondon *et al*. 2009).

1.4 AIMS

The formation of inimal hyperplasia in different vascular pathologies may be regarded as a physiological healing response in the intima aimed to repair the vessel after an injury, noxious stimuli or altered physical forces. Healing responses thus play a central role in such diverse conditions as in the formation of the fibrous cap in atherogenesis, in the repair of vulnerable lesions after plaque rupture, in restenosis after open surgical and endovascular interventions, in venous by-pass grafts, and in arteriopathy during chronic rejection of transplanted organs.

By increasing our understanding of the molecular pathways that regulate vessel wall repair, we can develop pharmacological methods to control SMC activation and proliferation to improve outcome after vascular surgery. Most importantly, the ability to pharmacologically regulate vessel wall healing should also enhance our possibilities to combat plaque instability and prevent the clinical consequences of atherosclerosis and plaque rupture.

Since vessel wall repair appears to constitute a fundamental process in vascular disease, investigations that encompass several different aspects of this pathology may create opportunities to improve translation of experimental data to clinical practice from bench to bedside.

The intentions with this project were therefore to evaluate processes underlying vessel wall repair using a broad range of strategies and experimental models such as

- molecular mechanisms involved in intimal hyperplasia with a focus on the role of IGF-1 in SMC proliferation (paper I)
- non-invasive imaging using high-resolution ultrasound to visualize rat carotid intimal hyperplasia (paper II)
- gene expression profiling of repair and stabilizing processes in human carotid atherosclerosis (paper III)
- the effect of hemodynamic forces on SMC gene expression *in vitro* and in the regulation of intimal repair (paper IV).

2 METHODS

2.1.1 in-vitro studies

2.1.1.1 Vascular smooth muscle and endothelial cell cultures

In order to study vessel wall biology in-vitro, we used SMC and EC cultures. SMC were isolated from rat aorta as described by Thyberg (Thyberg, Hedin *et al*. 1990). Aortas were dissected from adult Sprague-Dawley rats under sterile conditions and divided into small pieces, which were then placed in 0.1 % collagenase type II solution in 0.1 % BSA for 12 hours at room temperature. Cells were then filtered and washed before seeding on tissue culture plates. SMCs were cultured using media with 10 % fetal calf serum which leads to changes in cell phenotype from a contractile to synthetic state and these cells were easy to handle, prone to adhesion and growth (Roy, Tran *et al*. 2002). In the present studies we used secondary SMC cultures at passage 2 to 6.

Endothelial cells were obtained from human saphenous vein of patients undergoing bypass surgery for peripheral arterial disease. These cells were also isolated from the vessels by enzymatic digestion, but 0.1 % collagenase and 0.16 % dispase solution was applied only at luminal side of the vein for 20 minutes at 37°C. ECs were then cultured in media supplemented with 40% human serum, cholera toxin and isobutylmethylxanthine on plates covered with gelatin (Jansson, Bengtsson *et al*. 1998). For all experiments, cells were synchronized by serum deprivation.

2.1.1.2 Evaluation of proliferation and apoptosis in-vitro

The proliferation of cultured cells was assessed by counting number of cells after treatment or by DNA synthesis assays: 3[H]-thymidine or bromodeoxyuridine (BrdU) incorporation assays. While cell count gives a summarized evaluation of both proliferation and apoptosis, DNA synthesis assays characterize the S-phase of the cell cycle. In later experiments we preferred to use BrdU incorporation, as it does not require radioactive labelling.

For determination of apoptosis in cell culture, we used a flow cytometric Annexin V binding assay. SMCs positive for annexin V were regarded as undergoing apoptosis, and the subgroup of annexin V positive cells that were negative for propidium iodide were considered to be in early apoptosis.

2.1.1.3 In-vitro shear stress exposure

In order to study effects of blood flow on endothelial and SMCs in-vitro, several models have been proposed (Frangos, McIntire *et al*. 1988; Dardik, Chen *et al*. 2005). For our experiments, we chose a model described by Frangos and co-authors where cell culture media circulates over a relatively large surface covered with cell culture and flow is obtained by a hydrostatic gradient. Flow through the chamber is laminar with an approximate Reynolds number of 7.28 for a mean shear stress level of 14 dynes/cm2. Rat SMCs were grown on the plate under regular conditions until they became confluent when they were exposed for the laminar flow for 24 hours.

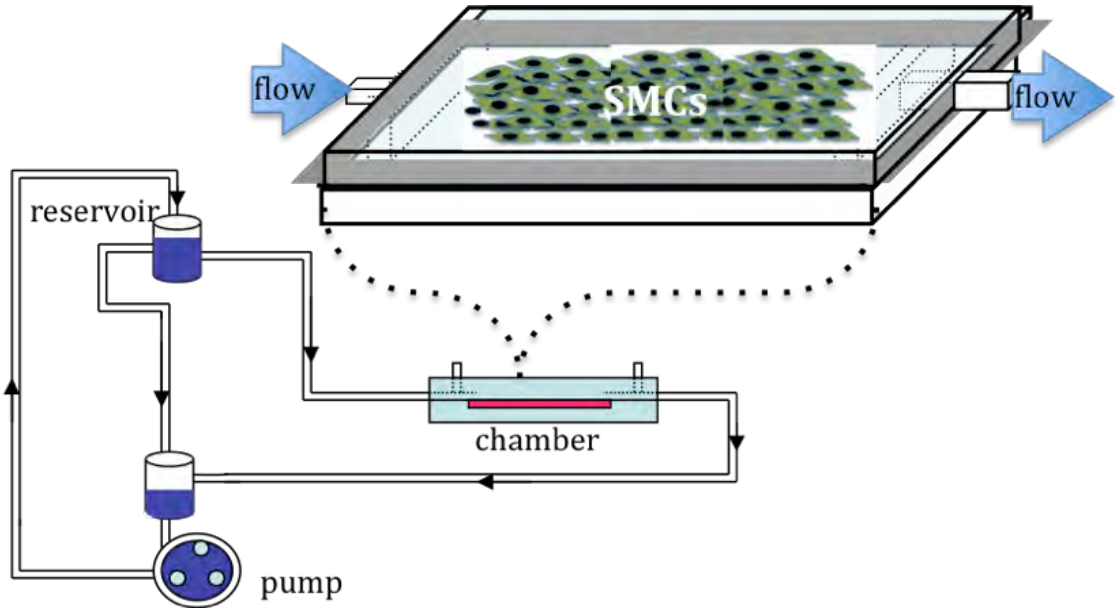


Figure 7. Model of the parallel plate flow chamber.

2.1.2 In-vivo experiments

2.1.2.1 Balloon injury of the rat carotid artery

For studying the healing process of the arterial wall in-vivo, we used a well-described model of intimal hyperplasia where the rat carotid artery is denuded by a Fogarty balloon catheter (Clowes, Clowes *et al*. 1989). In this model, medial SMCs are mechanically injured and exposed for activation signals from blood. When activated cells migrate on the luminal side of the internal elastic lamina, they proliferate and produce extracellular matrix. At the same time a slow process of reendothelisation commences and takes more than 4 weeks.

In paper I we used the model to study the effects of PPP on intimal hyperplasia, and the size of the neointima was assessed on histological sections stained with Masson-Trichrome. Micrographs of the cross sections were subjected to digital measurements of intimal area. In paper II we used the model to evaluate a new method of in-vivo visualization, ultrasound biomicroscopy. This required more advanced morphological analysis including measurements of intima, media and wall thickness, and calculation of lumen diameter.

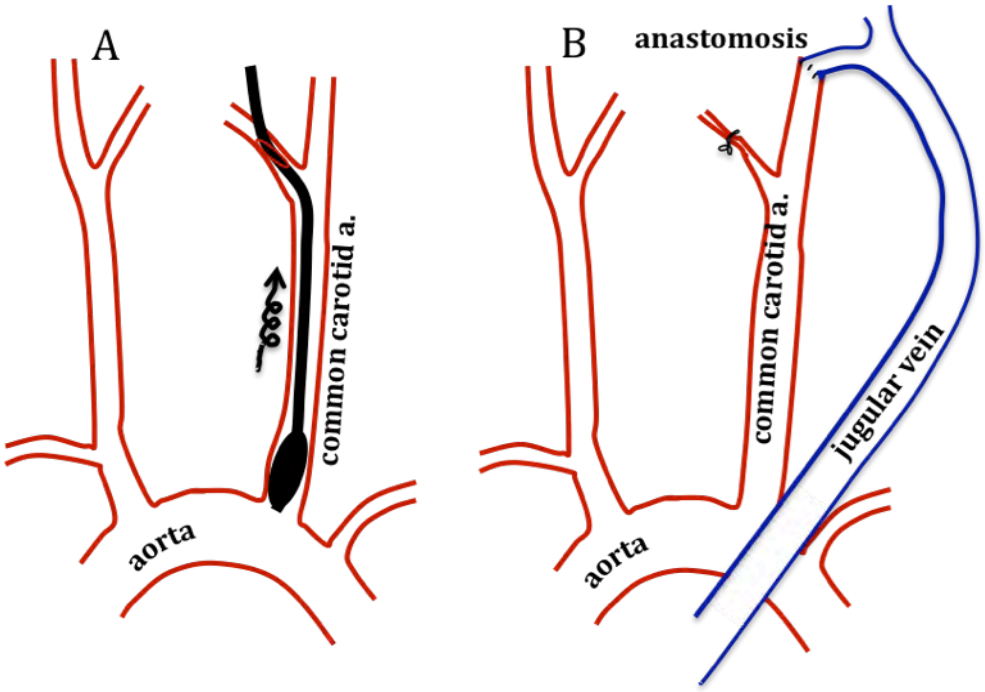


Figure 8: Rat carotid balloon injur model A: catheter is introduced through the external carotid artery and the endothelium denuded in the common carotid artery B: Arteriovenous fistula created between the internal carotid artery and the jugular vein to increase flow after balloon injury

2.1.2.2 Balloon injury and flow modification

We modified the rat carotid balloon injury model by creating an arterio-venous fistula between the internal carotid artery and the jugular vein and the anastomosis was created using 6 to 8 single sutures (11-0 prolene). This procedure led to a 4-fold increase in blood flow through the injured artery from 3.9 ± 0.5 to 18.2 ± 0.8 ml/min (unpublished data). Volume blood flow was calculated from measurements of lumen diameter and mean flow velocity assessed with ultrasound (Vevo 770 system).

2.1.2.3 Ultrasound biomicroscopy

In paper II we evaluated the method UBM for assessment of intimal hyperplasia after balloon injury of rat carotid artery. We used Vevo 770 system from Visualsonics (Canada) equipped with three probes: 30, 40 and 55 Mhz. This system is characterized by a very high spatial resolution (30 μm axial resolution with 55 MHz probe) achieved by high frequency ultrasound signal. Left and right common carotid arteries were visualized from the clavicle to the bifurcation. Anaesthesia is required to immobilise the animal and we found isoflurane inhalation to be very convenient for performing surgical procedures, ultrasound examinations and sacrificing the animals. Since rats, as other small animals, tend to have a large surface area-to-body-mass ratio and rapid body metabolism, they are prone to heat loss. To prevent this we use heating devices such as thermal heating pads. From each of the three levels (proximal, middle and distal), a cine loop of the B-mode image was saved. Measurements were performed off-line using built-in software on the Vevo 770 system. The measurements were done according to the leading edge principle (Wendelhag, Gustavsson *et al*. 1991) and the intima (IT), media (MT) and wall thickness as well as lumen diameter were measured. Lumen diameter was defined by the distance between the intima–lumen interface of the nearwall and the lumen–intima interface of the farwall. Intima–media thickness was defined as the distance from the lumen–intima interface to the media–adventitia interface. Intima was defined as the distance from the lumen–intima interface to the intima–media interface, and media from the intima–media interface to the media–adventitia interface (Fig. 9). The intima-to-media ratio was then calculated from the determined means of IT and MT. On the noninjured vessels as well as on the arteries with a small intimal layer, lumen diameter was measured as the distance between adventitia–media interface on the near wall and the lumen–intima interface on the far

wall, minus far wall IMT. Accuracy of these measurements was determined by comparing UBM measurements with morphometry data. In order to verify if UBM technique allows us to see real layers of the vessel wall we performed an experiment when rat arteries with partly dissected intima were visualized ex-vivo.

In paper IV we used the Vevo 770 system not only to visualize rat carotid arteries and measure lumen diameter, but also for determination of volume blood flow. We measured velocity-time index on histograms obtained with pulse waver Doppler mode. For flow assessment, we used the most proximal part of the common carotid artery where it is easier to achieve a correct angle due to the anatomy.

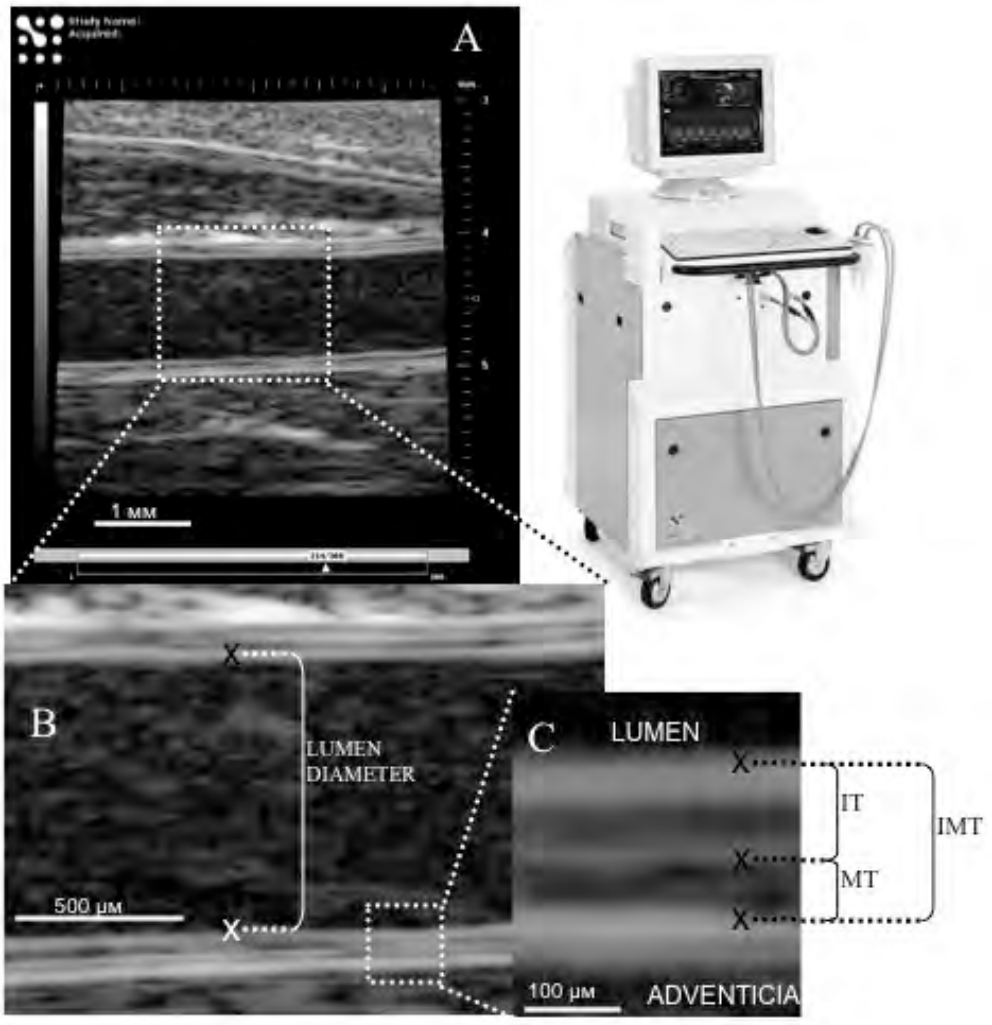


Figure 9: Rat common carotid artery 14 days following balloon injury. Visualization with 55 MHz probe on Vevo 770 system. Lumen diameter was defined by the distance between the intima–lumen interface of the nearwall and the lumen–intima interface of the farwall. Intima–media thickness was defined as the distance from the lumen–intima interface to the media–adventitia interface. Intima was defined as the distance from the lumen–intima interface to the intima–media interface, and media from the intima–media interface to the media–adventitia interface

2.1.2.4 Treatment with picropodophyllin

At the time we were evaluating potency of PPP to inhibit intimal hyperplasia, no attempts to use this drug in rats had been reported. We therefore performed a series of experiments in order to test pharmacokinetics of the compound in rats. We injected PPP intraperitoneally as 0.2 mL of a 100-mmol/L PPP (20 micromols) solution consisting of Dimethyl sulfoxide (DMSO) and sunflower oil (9:1). Blood samples were collected at different times points after injections and PPP concentration in plasma was determined by reversed-phase high-performance liquid chromatography. Treatment with 20 μmol of PPP (8 mg = 20 mg/kg body weight) every 12 hours was enough to keep the plasma concentration of PPP >200 nmol/L, a concentration previously found to inhibit cancer growth in mice (Girnita, Girnita *et al*. 2004). We used this administration regimen in further experiments. Animals in the control group received the same volume of DMSO/sunflower oil solution. Drugs were administered under isoflurane anesthesia every 12 hours. Injections were well tolerated by the animals for several days but after a longer time PPP caused irritation of the peritoneum. In an additional experiment we tested oral administration of PPP. The drug was given to the animals by gavage in the same amount as intraperitoneally twice a day for 2 weeks.

2.1.2.5 Immunohistochemistry

Immunohistochemical staining of rat carotid arteries was used to assess SMC proliferation and apoptosis in-vivo. In paper I we used antibodies against proliferating cell nuclear antigen and caspase-3 respectively. Immunostaining was also used in paper IV to evaluate the expression of tissue factor pathway inhibitor 2 (TFPI-2) on protein level as well as its' localisation in the injured artery in relation to cells expressing SMC alpha-actin and von-Willebrand factor.

2.1.3 Protein determination

In order to evaluate presence and relative amount of proteins, Western blotts were performed. In contrast to immunohistochemistry, this method does not specify localisation of the molecule in relation to cellular or tissue structures but allows studying proteins and their posttranslational modification which may be hard to identify in situ. The method requires tissue homogenisation and cell lysis. By means of electrophoresis the extracts are separated according to protein mass and molecules of interest are

identified using specific antibodies. We used Western blotting in paper I to estimate the ability of PPP to block phosphorylation of IGFR-1. Immunoprecipitation technique was used to evaluate amounts of phospholylated IGFR-1 in cultured SMCs and in injured rat carotid arteries after treatment with PPP. The same material was analysed for levels of total and phosphorylated Akt.

In paper IV, the amount of TFPI-2 was determined with Western blotting in SMC cultures exposed for fluid shear stress for different time periods.

2.1.4 mRNA expression analysis

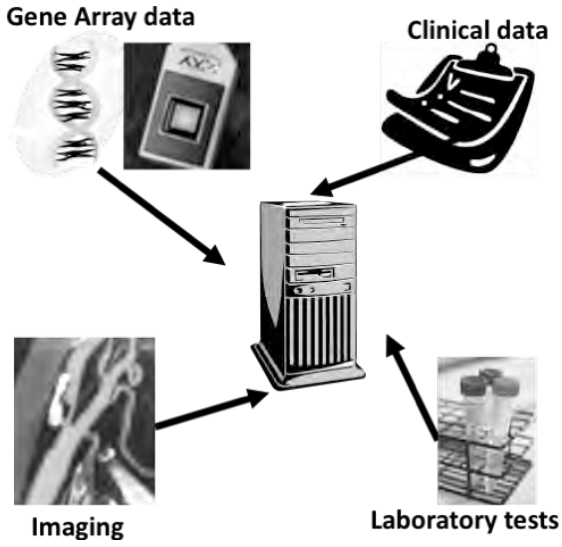
Activity of gene translation can be evaluated by assessing the amount of messenger RNA. To determine the expression of a gene, quantitative real time reverse transcription PCR is widely used. PCR is preceded by a reaction using reverse transcriptase to convert RNA to cDNA and to measure the amount of amplified product in real time (Kramer and Coen 2006).

Another possibility is to study expression of thousands of genes at once, and thereby create a global picture of cellular function by utilising gene expression profiling based on DNA Microarray technology (Microarrays, Science Primer, 2007). This method measures the relative activity of previously identified target genes. Similar to real time PCR method, gene microarrays require isolation of total mRNA from a sample. We used Affymetrix's GeneChips technology to perform global gene expression analysis of cultured SMCs, injured rat carotid arteries and human atherosclerotic plaques.

In paper IV microarray analysis of cultured SMC was performed in order to identify candidate genes responsible for phenotypic changes of cells being exposed to fluid shear stress. Quantitative real time polymerase chain reaction was used to confirm the expression data generated by microarrays.

In paper III we utilized DNA microarray technology to study relationships between gene expression patterns in human carotid plaques and clinical variables associated with plaque instability. As the technology is progressively developing, it provides reproducible data and it has been suggested that the results may be used without further validation (Couzin 2006), especially when such verification is impractical due to a large number of genes studied at the same time. Array data from BiKE has also been previously confirmed with good accuracy using real time PCR in a number of studies (Qiu, Gabrielsen *et al*. 2006; Tran, Agardh *et al*. 2007; Malarstig, Sigurdsson *et al*. 2008; Olofsson, Soderstrom *et al*. 2008; Olofsson, Soderstrom *et al*. 2009)).

2.1.5 Biobank of Karolinska Carotid Endarterectomies



The Biobank of Karolinska Carotid Endarterectomies (BiKE) is a research project which was established in 2003, in collaboration between the Department of Vascular Surgery at Karolinska University Hospital and Cardiovascular research groups at Karolinska Institutet (Experimental Cardiovascular Group at the Center for Molecular Medicine and the Department of Molecular Medicine and Surgery).

In order to better understand the pathogenetic mechanisms underlying atherothrombosis in genereal and the clinical manifestations of cerebrovascular disease in particular, we need to identify molecular mediators expressed in the pathologic human tissue.. Hypothesis-driven analysis has prevailed but modern molecular medicine offers exciting opportunities for unbiased exploration of gene expression. By isolating RNA from lesions, reverse-transcribing it to cDNA, and hybridizing to “global” oligonucleotide arrays, the total expression pattern of a pathological tissue can be deduced. This approach is becoming widely used, however the simultaneous analysis of tens of thousands of genes poses novel problems of statistical nature. Since multiple comparisons are made, it is not sufficient to analyse a few samples. In addition, genetic individuality creates a variability that can only be handled by increasing the sample size. With a large enough study group of patients, a DNA biobank, and a clinical database, it would even be possible to identify individual patterns of gene expression linked to genetic polymorphism or specific environmental factors.

Since 2003 all atherosclerotic lesions obtained at surgery for asymptomatic and symptomatic carotid stenosis in the mentioned clinic are collected and stored. The larger part of the plaque is then used for total RNA isolation and is subjected to global gene expression array. From the same patients samples of plasma and white blood cells are taken at the time of surgery. A broad range of clinical information about the patients including laboratory tests, co-morbidity and visualisation data, are saved in a database which is then linked to the data from gene expression arrays.

The BiKE contains currently more than 450 atherosclerotic lesions, 150 carotid endarterectomies have been subjected to gene expression array and a number of studies, which utilize the material from the biobank have been pulished (Qiu, Gabrielsen *et al.* 2006; Tran, Agardh *et al.* 2007; Malarstig, Sigurdsson *et al.* 2008; Olofsson, Soderstrom *et al.* 2008; Olofsson, Soderstrom *et al.* 2009)).

2.1.6 Human atherosclerotic specimens

Material described in paper III is a part of the BiKE project and includes atherosclerotic lesions obtained from 106 patients who underwent TEA for carotid stenosis (Mean age 70.0 years, range 46-85 years, 85 males and 21 females). Five samples of iliac arteries obtained from organ donors were included in the study as controls. We registered the following clinical variables: presence and type of symptoms, time between the last symptom and surgery, statin treatment and pre-operative duplex ultrasound assessment of carotid plaque echodensity.

Endarterectomy speciments were taken at surgery and snap frozen with subsequent total mRNA extraction. The RNA was used for gene expression profiling using Affymetrix HG-U133 plus 2.0 A Genechip® arrays from Affymetrix (www.affymetrix.com).

Out of more than 22 000 genes we selected over 300 genes representing proteins associated with either plaque instability or healing processes in the vessel wall through a Pub-Med reference-based biased approach. Each protein was assigned to a function code, which was used for sorting, and all genes were divided into 4 main functional groups according the role they presumably play in atherosclerotic plaques. The four groups were given color codes to indicate general function.

Expression levels for the probe sets included in our prior hypothesis were exported and further analyzed. In cases where one gene had two or more probes sets the one marked "_at" was used in preference of "_s_at" probe sets. "–x_at"- labeled probe sets were used only when neither "_at" or "s_at" probe sets were present (GeneChip® Expression Analysis. Data Analysis Fundamentals). If two or more of same probe set suffix type were present, the probe set with higher expression values was chosen. Patients were then grouped according to the clinical variables described above. The median value per group was calculated and used for the calculation of group-to-group ratios. All 318 genes were than sorted according to the ratio in order to identify genes differentially expressed in the groups of interest.

Table 2. Gene classification.

Green	• SMC activation and proliferation • anti-inflammatory • anti-apoptotic • MMP inhibition • SMC markers
Light-green	• ECM components and production • cytoskeletal • cell-matrix interactions • endothelial cell markers
Orange	• cell adhesion • lipid metabolism • coagulation • angiogenesis
Red	• pro-inflammatory • pro-apoptitic • anti-proliferative • ECM degradation • inflammatory cell markers

2.1.7 Statistical analysis

When analyzing differences between the histomorphometric data of the two groups, the two-sided Student t test was used and the results were presented as mean ± the standard deviation. To compare multiple groups, the data were analyzed with one-way analysis of variance and the Bonferroni multiple comparison test. In paper II we performed correlation analysis and Pearson correlation coefficients were computed to illustrate the relationship between the ultrasound-assessed and histology values. Bland–Altman test was used to show individual variations compared to the average values from two methods of IMT estimation. We evaluated intraobserver reproducibility for measurements performed on histology and UBM in terms of coefficients of variation. In paper III, significance was evaluated by one-way ANOVA, Kruskal-Wallis test or unpaired t test with Welch's correction when appropriate. In the last paper Mann-Whitney U test was used for statistical significance. *P*-values for microarray data in paper IV were calculated using the Wilcoxon Signed Rank Test and Tukey Biweight.

Fold change was calculated using the signal log ratio of the different experimental conditions according to Affymetrix gene chip analysis methodology. Statistical significance for real-time PCR data was assessed using a 99% confidence interval for the standard error of the mean. A *p*-value of less than 0.05 was considered significant.

For the statistical calculations and drawing of the diagrams, GraphPad Prism (Graph Pad Software, Inc. Systems, San Diego, CA) and Statistica version 6 (StatSoft, Inc. Johannesburg, South Africa) software were used.

3 RESULTS AND DICUSSION

3.1 PAPER I. ATTENUATION OF INTIMAL HYPERPLASIA WITH A SELECTIVE INHIBITOR OF THE IGF-1 RECEPTOR

IGF1 plays a critical role in vascular repair by regulating SMC behaviour in response to injury. In paper I we investigated a possibility to block IGF-dependent signaling by specific pharmacological targeting of IGF1R in vascular SMCs. Furthermore, the role of IGF1R in formation of intimal hyperplasia was studied in the rat carotid balloon injury model. Rats were treated intraperitoneally with an inhibitor of IGF1R phosphorylation – picropodophyllin at a dose 40 μmol/day for two weeks after the injury. Histological evaluation of neointima size showed that PPP inhibited intimal hyperplasia by 50%. We did not observe any toxic effects of the treatment but repetitive injections of the compound caused irritation of the peritoneum. Another group of animals were given PPP in the same dose by gavage for two weeks following injury. Similar results with considerable reduction of intimal hyperplasia were achieved with this type of delivery (unpublished data, Figure 10).

Using immunostaining we determined proliferation activity of SMCs in the rat carotid artery 4 days after balloon injury and found that PPP decreased the number of cells positive for PCNA. Staining for activated caspase 3 showed very few positive cells in both the control and PPP groups suggesting low levels of apoptosis 4 days after the injury. Further evaluation of IGF1R signaling in SMCs was performed *in vitro*. Treatment with PPP significantly decreased cell number in cultures stimulated with IGF-1, and had less pronounced inhibitory effect on SMC activated by serum. The fact that the IGF1R inhibitor suppressed growth of the cells stimulated by serum, which contains a variety of other growth factors in addition to IGF-l, suggests that some of these may act through the IGF-l pathway either by transactivation of IGF-lR or by stimulating synthesis and release of IGF-l (Delafontaine, Anwar *et al*. 1996; Scheidegger, Du *et al*. 1999; Du, Brink *et al*. 2001; Frederick and Wood 2004). DNA synthesis and annexin V binding assays showed that the inhibitory effects of PPP on SMCs were mainly due to abolished proliferation activity and cannot be primarily attributed to apoptosis. Taken together with the decreased number of PCNA cells, these results propose that PPP affects the G1-S phase transition in proliferating SMCs.

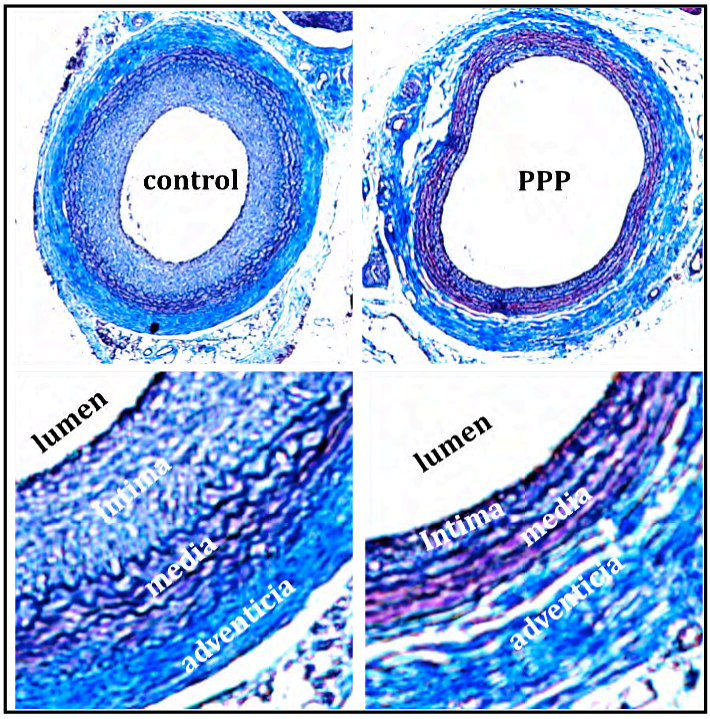


Figure 10. Effect of oral treatment with PPP for two weeks on the development of intimal hyperplasia in rats after balloon injury. Masson's trichrome staining.

In order to elucidate which intracellular mechanisms were affected by PPP treatment we determined phosphorylation of IGF1R and Akt, a member of the PI-3 kinase/Akt pathway and which is a major signaling cascade activated by IGF-1. We found that PPP treatment attenuated phosphorylation of the receptor and downstream target Akt in cultured SMCs activated by IGF-1 as well as in rat carotid arteries where phosphorylation levels were increased by the injury. Signaling through MAPK/ERK (extracellular signal-regulated kinase) was also decreased as demonstrated by decreased ERK1/2 phosphorylation. These findings are in line with previous reports demonstrating that PPP can decrease ERK activation in IGF-l–stimulated cell lines (Vasilcanu, Weng *et al*. 2006).

The specificity of PPP for IGF1R was assessed when SMCs activated with PDGF were treated with PPP. In this experiment PPP did not affect Akt phosphorylation, whereas wortmannin, an inhibitor of the PI-3 kinase pathway, blocked Akt activation after PDGF stimulation. Phosphorylation of ERK1/2 was caused by both IGF-1 and PDGF; however, IGF-1 stimulation led to a shorter and less pronounced activation compared with PDGF. Addition of PPP to the cell culture medium decreased ERK1/2 activation in cells treated with IGF-1 but not with PDGF. In light of these findings, we may propose that, despite the suppressed proliferation in SMCs activated by serum, PPP does not interfere with the early signaling mechanisms initiated by PDGF, despite the suppressed proliferation in SMCs activated by serum. However, this does not exclude later effects

mediated through increased autocrine IGF-l synthesis (Delafontaine, Lou *et al*. 1991; Ververis, Ku *et al*. 1993).

We could show that pharmacotherapy specifically targeting the IGF-l axis can attenuate intimal hyperplasia after balloon injury. Treatment with PPP suppressed SMC proliferation by inhibition of IGF-1R phosphorylation and IGF-1R–mediated downstream signaling.

The study supports the fact that SMC proliferation is a key element in the healing reactions on vessel wall injury, and that IGF-1 signaling has an important role in intimal hyperplasia by serving as a potent SMC mitogen.

3.2 PAPER II. VISUALISATION OF RAT NEOINTIMA WITH ULTRASOUND BIOMICROSCOPY

As described in paper I, the rat carotid balloon injury model is broadly used in our laboratory to study mechanisms of intimal hyperplasia. In order to estimate the size of the neointima, cross sections of the artery are usually prepared and used for digital morphometry. Although this is a very well established method, it requires time-consuming histological preparations and requires the sacrifice of animals at each time point of analysis. For that reason we were interested to establish a method of *in vivo* assessment of structural changes in the vessel wall in rats. Recently, a number of encouraging papers regarding high frequency ultrasound have been published and we set out to evaluate if this method would be the right technique to fulfil our needs (Martin-McNulty, Vincelette *et al*. 2005; Gan, Gronros *et al*. 2007; Goldberg, Pakkiri *et al*. 2007). Several pilot examinations were performed of rat arteries with Vevo 770 system (Visualsonics, Canada) and we observed that the structure of normal and injured carotid arteries could be reproducibly visualised and the wall thickness measurable with this method. The injured artery was represented on B-mode image as three lines as described by Osika *et al* for human radial arteries in elderly individuals (Osika, Dangardt *et al*. 2007). They characterized a new concept in the vascular ultrasound field since it had previously only been possible to determine the intima-media complex.

In order to verify which structures of the vessel wall were represented on the UBM picture, we did a series of ex vivo experiments where arteries with partially denuded intima were visualized. The disrupted intimal layer was used as a reference to compare pictures obtained by UBM with those from histology. This approach had previously been applied for visualization of human arteries by Pignoli *et al* and Wendelhag *et al* (Pignoli, Tremoli *et al*. 1986; Wendelhag, Gustavsson *et al*. 1991). We discovered that

the three-line wall structure seen on the UBM picture represents the actual histological composition of the vessel: the first line stands for the interface between the blood and intima, while the second one represents the internal elastic lamina (between the intima and media), and the third one the external elastic lamina (between media and adventitia).

The reliability of the measurements performed *in vivo* by UBM was compared to those made on histological sections. We compared measurements of intima-media, intima and media thickness as well as lumen diameter assessed with UBM and morphometry on the intact or injured arteries. UBM was found to slightly overestimate IMT compared with morphometry, as previously shown by Gan *et al* in ApoE knockout mice measuring plaque thickness (Gan, Gronros *et al*. 2007). The difference can be explained by the tissue shrinkage during the fixation process as well as by the absence of vessel tone. Further analysis revealed a good correlation between IMT assessed by UBM and morphology. While measuring more tiny structures as MT, we found a weak correlation between ultrasound and histology. This observation can be explained by the limitations in spatial resolution which is 30 μm for the system used in the study while MT is about 50 μm. Tunika media is also the layer which is affected most severely by post-mortem changes due to disappearance of blood pressure and vascular tone. At the same time, measurements of IT on UBM correlated significantly with histology, most likely because the neointima is thicker than the media, varies more in size and displays less postmortem changes. Importantly hisotological determination of intimal area (IA), which is the most frequently used parameter to characterize intimal hyperplasia, correlated significantly with IMT on UBM. This may be explained by the fact that MT remains almost the same after balloon injury and changes in IMT are due to the changes in IT. We believe that these results can permit us to use IMT as an indirect measurement for IT, especially at earlier time points, when intima and media are hardly distinguishable from each other with UBM.We also evaluated the intraobserver variability for histological and UBM measurements. The coefficient of variation was higher for UMB than for assessment of intima and media areas on histology and many factors can contribute to that. We consider the coefficient of variation of 11% to be sufficient for the assessment of intima thickness and 8% for IMT on a living rat. Especially if the variation is much lower than the pharmacological treatment effect noted.

The great advantage of *in vivo* visualization is the possibility to use the same animals for assessment of same parameters at different time points, which conforms to the

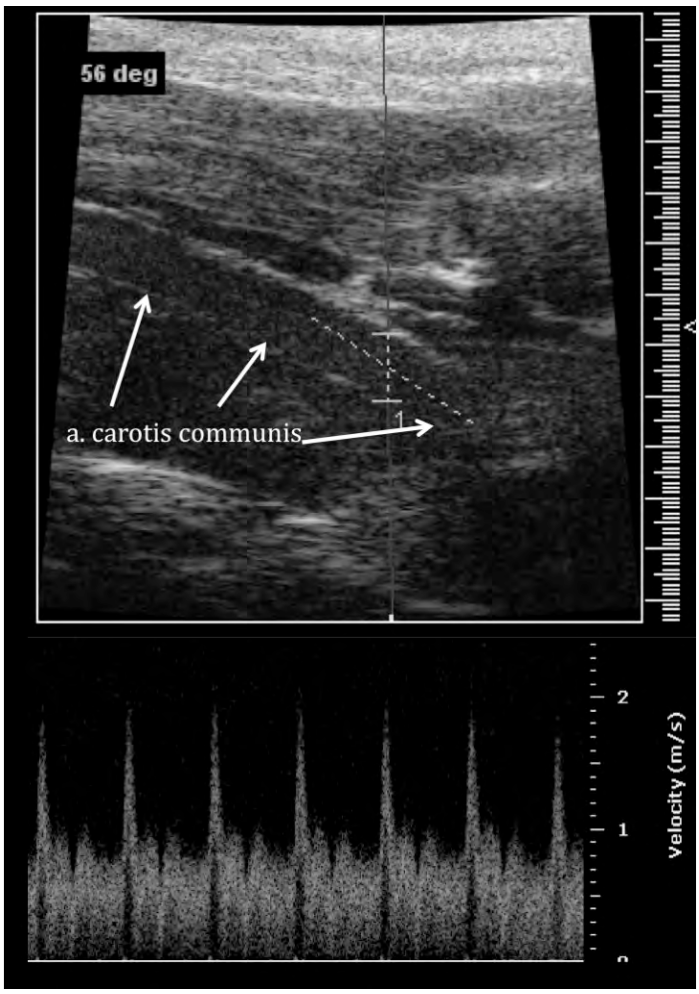


Figure 11. Assessment of blood flow in the proximal segment of rat common carotid artery after creation of an arteriovenous fistula between ICA and juguar vein. Visualization with 40 MHz probe on Vevo 770 system.

"reduction" principle – one of the "three R" principles of animal care formulated by Russel (Russell 1995). We detected thickening of the vessel wall from the first day after the injury, which continued during 2 weeks with following stabilization. We also found that increase of vessel wall thickness over time was followed by decrease in the lumen diameter. *In vivo* assessment of LD in the rat model and in real time provides new ways to study arterial remodeling and flow effects on the structure and function of the vessel wall. In this aspect UBM is in fact superior to histomorphology since postmortem changes as well as histological preparation make it almost impossible to accurately evaluate LD.

In order to evaluate the practical applicability of UBM, we used PPP to inhibit intimal growth in a part of the animals used in this study. Measurements of IMT by UBM and morphometry 2 weeks after balloon injury showed that the effect of PPP treatment could be evaluated by ultrasound. Indeed IT is not the only parameter to evaluate when studying the effect of pharmacological agents on intimal hyperplasia formation. However, *in vivo* estimation of the arterial wall thickness using UBM provides an

effective and rapid way to study the effects of new drugs in this field (Liang and Blomley 2003).

After the completion of this study, we have continued using UBM in our experimental work. Apart from estimation of intimal hyperplasia we utilize the system to assess blood flow in the rat carotid artery after creation of an arteriovenous fistula and for evaluation of aorta dimension in animals with experimental abdominal aortic aneurysms. In the last model UBM permits excellent visualisation of the complex anatomy of the aneurysm and measuring both inner and outer diameter of the aorta under physiological conditions are feasible. In the animals, where we created an arteriovenous fistula between the external carotid artery and the jugular vein we evaluated volume flow using measurements of lumen diameter and flow velocity. In our experiments (unpublished data) arteriovenous fistula creation caused a 4-fold increase in volume blood flow in CCA (from 3.9 ± 0.5 to 18.2 ± 0.8 ml/min). This resulted in inhibition of intimal hyperplasia (IMT from 140.3 ± 17.2 μm to 91.5 ± 9.9 μm). Interestingly, a 4-fold change in blood flow did not cause outward remodelling of the artery and the increase in arterial diameter was due to less advanced intimal hyperplasia in the common carotid artery (CCA). Diameter of the CCA was 0.84 ± 0.06 mm in sham group and 1.1 ± 0.05 mm in CCA with increased flow.

To summarize, in paper II we applied the UBM method to the rat model of intimal hyperplasia and found it possible to visualise intact or injured common carotid artery structure. We could show that this technique is sufficient to accurately monitor the healing reaction of the injured artery and to assess an effect of pharmacological inhibition of neointima formation. UBM technique has a great potential to become a common and important tool of experimental research in the field of vascular biology and will contribute to the understanding of pathophysiological mechanisms of cardiovascular disease.

3.3 PAPER III. GENE EXPRESSION PROFILES IN CAROTID PLAQUE INSTABILITY AND HEALING

In the third paper we analyzed gene expression microarrays from over one hundred carotid plaques and showed that clinical variables reflecting lesion instability correlate with mRNA expression profiles of genes previously proposed to be involved in destabilizing or stabilizing processes in the vessel wall.

We found remarkable differences in gene expression levels between the normal artery wall and lesions. 82 genes were more than 10% upregulated in atheroma, several showed a 10 fold increase in expression and among those mRNA levels for MMP-9 and MMP-12 were found to be more than 100 fold higher in plaques. In agreement with previous reports, some other proteases and inflammatory mediators were also overexpressed in lesions (Galis, Sukhova *et al*. 1994; Choudhary, Higgins *et al*. 2006; Higashikata, Yamagishi *et al*. 2006). 71 genes were more than 10% downregulated in plaques, expression levels of 4 genes were decreased more than 10 times (musculoskeletal embryonic nuclear protein 1, phospholipase A2, Filamin- C, smooth muscle-actin g 2). Samples of healthy iliac arteries were thereby characterized by higher expression of SMC specific genes while levels of the macrophage marker CD68 were lower. This confirms the difference in cellular content between a normal arterial media and a diseased and inflammatory intima.

In contrast to the data presented above, further comparisons were made between atherosclerotic lesions grouped according to various clinical parameters and thereby did not show such outstanding discrepancies in expression profiles as it was observed between plaques and healthy arteries.

Comparisons between asymptomatic and symptomatic plaques demonstrated that 6 genes were significantly downregulated in lesions from symptomatic patients (PDGF-D, PDGFR b, collagen IV alpha 6, osteoglycin, growth arrest-specific 6, tropomyosin b), while mRNA levels of 14 genes were increased (MMP-9, MMP-12, VEGF, ADAM 8, tenascin C, IGF-1BP1, IL-1 receptor-associated kinase 1, IL-6, integrin alpha 5, TIMP-1, urokinase plasminogen activator, PAI-1, thromboxane A synthase 1, osteopontin). These data represent a trend towards overexpression of genes related to inflammation such as MMPs in symptomatic and unstable lesions whereas asymptomatic plaques had higher levels of mRNA coding for extracellular matrix proteins and SMC markers. Previously Loftus *et al* showed that MMP-9 expression was higher in symptomatic lesions (Loftus, Naylor *et al*. 2000) and a number of other studies described increased

matrix degradation in vulnerable plaques (Morgan, Rerkasem *et al*. 2004; Choudhary, Higgins *et al*. 2006; Higashikata, Yamagishi *et al*. 2006). In other reports symptomatic carotid plaques were present with higher angiogenic activity and overexpression of CD 163 (Tureyen, Vemuganti *et al*. 2006; Ijas, Nuotio *et al*. 2007).

Another variable we analyzed in relation to the gene expression profiles was type of symptom of cerebrovascular disease. Patients with TIA or MS were compared with patients who had suffered from amaurosis fugax only. It has been suggested that patients with MS or TIA have higher stroke risk than subjects with retinal events (Naylor, Rothwell *et al*. 2003; Rothwell, Eliasziw *et al*. 2004). Morphological analysis of endarterectomy samples carried by Verhoeven *et al* revealed structural similarities between lesions from asymptomatic patients and subjects with amaurosis fugax (AF) and it has been proposed that AF may be caused by microembolisation from discrete erosions rather than complete atheroma ruptures with thrombosis (Benavente, Eliasziw *et al*. 2001; Verhoeven, Hellings *et al*. 2005). AF has also been suggested to be caused by lesions in the common and external carotid arteries or advanced lesions in the internal carotid artery (ICA) involving the ocular artery as a way of collateral blood supply (Hoya, Morikawa *et al*. 2008).

In our material gene expression levels did not differ significantly between plaques from patients with AF and lesions from individuals with MS or TIA. In the list of 318 genes included in the study, two genes were significantly upregulated (apolipoprotein E and integrin alpha M) in plaques from patients with MS and TIA whereas only PI3 kinase expression was significantly lower. However, an overall evaluation of gene expression suggests that plaques from patients with AF are characterized by dominant expression of genes related to plaque stabilization, which may support the theory that ocular symptoms of cerebrovascular disease are rarely caused by plaque rupture.

Considering the clinical evidence that the risk of stroke gradually subsides over time in patients with symptomatic carotid stenosis (Redgrave, Lovett *et al*. 2006; Rothwell, Giles *et al*. 2007) we assumed a time-dependent change in plaque phenotype with stabilization after possible rupture.

Evaluation of mRNA levels in relation to the time between the last qualifying symptom of plaque instability (MS, TIA or AF) and surgery revealed that this variable influenced gene expression profiles. We divided all symptomatic lesions in to four groups: those operated within the first 14 days, between 15 and 30 days, between 31 to 90 days, and more than 90 days after the last embolic event. The comparison between the first group (within 15 days) and the third one (31 to 90 days) showed intriguing patterns, although

only few genes with significant difference in expression were found. Functional classification of genes suggests that lesions removed early after symptoms of plaque instability are characterized by less inflammation and proteolysis. In addition, various SMC marker genes were clustered in lesions with short symptom-free interval, while levels of macrophage and T-cell marker genes were lower. These findings imply that plaque rupture may induce a rapid healing reaction with activation of SMCs that lead to plaque stabilization over time. We also observed a trend of gene expression levels either increasing (for example TNF-alpha) or decreasing (for example myocardin) with time but returning to levels near those observed at the early time point. This observation may be influenced by the fact that individuals with relatively large infarctions, possibly caused by prominent plaque rupture and more massive thrombosis, may be subjected to delayed intervention and therefore prevail in the group of patients operated later than 2 weeks after stroke (Eckstein, Schumacher *et al*. 1998; Virmani, Burke *et al*. 2006).

The tendency of the mRNA levels to return back and have a higher variability may be a sign of the cyclic process of atheroma progression, when the healing response triggered by rupture is replaced by a new wave of plaque degradation. This trend may partly explain the contradiction between our findings and a number of studies, which have shown abundance in macrophages and increased proteolysis in lesions obtained in the early phase after embolic symptoms (Redgrave, Lovett *et al*. 2006; Peeters, Hellings *et al*. 2009). Those studies compared plaques obtained within the first month after the last embolic event with those obtained later than 3 months while our paper is the first to our knowledge to analyze lesions obtained within 14 days after an event as a separate group. Another reason could be that these investigations used histomorphology while we assessed gene expression levels.

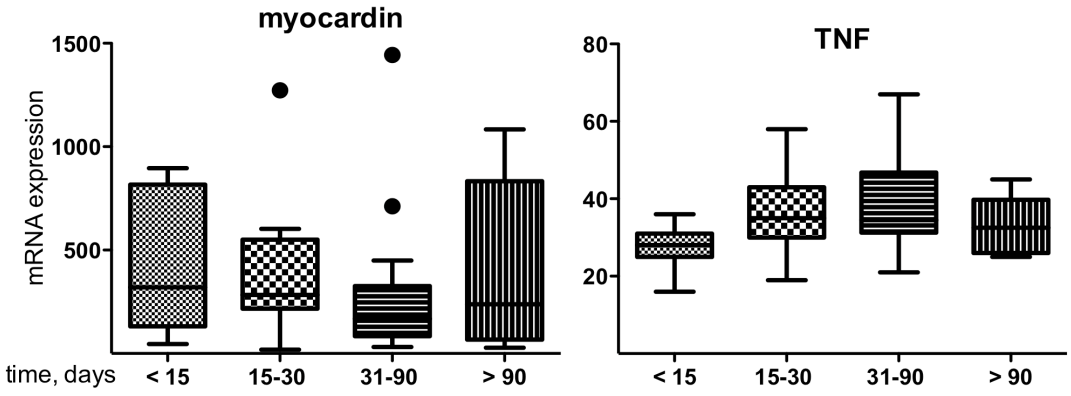


Figure 12. mRNA expression of myocardin and TNF-alpha in relation to time since the last qualifying symptom.

It is important to note that the very first two weeks after a minor stroke or TIA are associated with most of the risk burden according to a number of studies (Coull, Lovett *et al*. 2004; Rothwell, Eliasziw *et al*. 2004; Rothwell, Giles *et al*. 2007). Another reason could be that these investigations used histomorphology while we assessed gene expression levels.

Decreased macrophage infiltration without change in MMP-2 -8 and -9 activity with time from ischemic event was reported recently by Peeters *et al*, suggesting that MMPs are produced by SMCs at later time points (Peeters, Hellings *et al*. 2009). In our material we found, in contrast, an inverse correlation between expression of SMC marker genes and MMP-7 -9 and -12 rather implying inflammation dependent MMP expression.

Use of statins has been shown to decrease risk of cerebral ischemic events in a number of clinical trials (Baigent, Keech *et al*. 2005; Amarenco, Benavente *et al*. 2009). Morphological studies have on the other hand demonstrated a decrease in macrophage infiltration and a reduction of MMP-9 in carotid lesions by preoperative statin treatment. (Crisby, Nordin-Fredriksson *et al*. 2001; Baigent, Keech *et al*. 2005; Kunte, Amberger *et al*. 2008; Amarenco, Benavente *et al*. 2009).

In our material we observed that patients on statin treatment rather had increased expression of a large number of genes, such as von-Willebrand factor and elastin, while few were suppressed. Expression of the genes specific for SMCs or proteases seemed to be unaffected by statin treatment. These findings imply that statins may influence plaque stability by promoting expression of extracellular matrix genes in addition to their anti-inflammatory features (Mitani, Egashira *et al*. 2003). Altogether, these results may support our interpretation that statin treatment directly influences gene expression in the atheroma. Interestingly, we also found increased expression of geranylgeranylpyrophosphate synthase in plaques from statin-treated patients, suggesting a compensatory response to inhibition of HMG-CoA reductase.

Several morphological features of an atherosclerotic plaque can be visualized using ultrasound technique (Kwee, van Oostenbrugge *et al*. 2008). Some of these features such as size of the necrotic core, degree of neovascularisation and fibrous cap thickness are related to plaque instability and patients with echolucent plaques have previously been shown to carry an increased stroke-risk (Gronholdt, Nordestgaard *et al*. 2001; Libby 2008). Plaque echodensity as it was assessed during the preoperative duplex ultrasound routine examination in a portion of the patients was used in his study as a marker of plaque stability. In support of the previous reports, we observed that echolucent plaques had higher expression of genes related to inflammation and

proteolysis, while increased expression of SMC-specific genes and genes for extracellular matrix proteins were observed in echodense lesions.

Being inspired by the fact that plaque echomorphology qualitatively evaluated during a routine clinical examination was reflected by changes in gene expression we have initiated a study where both quantitative and qualitative parameters of plaque morphology (such as: grey scale mean, presence of local echolucent area, surface features and heterogenecity) are used. Our preliminary results, which include retrospective assessment of 32 lesions in the BiKE project, revealed significant correlation between GSM value and mRNA levels of IGF-1 ($p=0.026$), and fibronectin-1 ($p=0.014$). These proteins, known to promote vascular SMCs proliferation, are abundantly expressed in fibrotic lesions. Moreover activation of SMC has been attributed to the healing response in atherosclerotic lesion (Hedin, Roy *et al*. 2004; Razuvaev, Henderson *et al*. 2007; Chiang, Korshunov *et al*. 2009). Moreover activation of SMC has been attributed to the healing response in atherosclerotic lesion. This finding supports the view that echomorphological features may be helpful for evaluation of the repair process in human carotid plaques.

Among the 318 selected genes analyzed in this study, the expression of several genes appeared to be more influenced by the clinical variables than others. Furthermore the expression of these genes was affected in similar manner in the different comparisons presented above (table 4). Expression of MMPs 7, 9 and 12 and the macrophage marker CD68 were commonly up-regulated in samples with clinical parameters indicating plaque instability whereas expression of elastin, myocardin and myosin heavy chain was more frequently found to correlate with clinical estimates of stable lesions (Table 3). The data confirm that a balance between synthesis of extracellular matrix by SMCs and degradation by inflammatory cells lies behind clinical manifestations of carotid atherosclerosis and should be seen as a target for therapeutic strategies.

Table 3. Summary of genes commonly dependent on clinical variables used in the study. (* p≤0,05)

Gene	Plaques vs Controls	Symptomatic vs Asymptomatic	Stroke/TIA vs AF	Distant vs Recent	No statins vs Statins	Echolucent vs Echodense
MMP7	↑*	↑	↑	↑	-	↑*
CD68	↑*	↑	↑	↑	-	-
integrin, alpha X	↑*	↑	↑	↑	-	-
MMP12	↑*	↑*	-	↑	↓	↑
MMP9	↑*	↑*	-	↑	↓	↑
ADAM8	↑*	↑*	↑	-	-	-
carbohydrate (chondroitin 4)	-	↑	↑	-	-	↑
cathepsin D	-	↑	↑	↑	-	-
elastin	↓*	↓	↓	↓	↓*	-
myocardin	↓*	↓	↓	↓	-	↓
growth arrest-specific 6	-	↓*	↓	↓	-	↓
IGFBP6	↓*	↓	↓	-	-	↓
myosin, heavy chain 11	↓*	↓	-	↓	↓	-
PDZ and LIM domain 7	-	↓	↓	↓	-	↓
tropomyosin 2	↓*	↓*	-	↓	-	↓*

3.4 PAPER IV. EFFECTS OF FLUID SHEAR STRESS ON VASCULAR SMC

As discussed earlier SMCs may be exposed to blood flow after various interventional procedures to the vessel wall when the endothelial layer is denuded. Not only molecular signals from blood but also physical forces reach SMCs especially those which form the neointimal layer. Hemodynamic factors can affect phenotypic modulation of SMC, promote intimal hyperplasia or cause vascular atrophy (Min, Kenagy *et al*. 2008). In paper IV we performed a series of *in vitro* and *in vivo* experiments where SMCs were exposed to fluid shear stress in order to dissect mechanisms underlying effects of the hemodynamic forces on these cells.

Using a specially designed flow chamber, we exposed secondary SMCs to FSS at arterial levels (14 dynes/cm^2) for 24 hours. Proliferation as assessed by BrdU incorporation in SMCs exposed to FSS was strongly decreased in line with previous reports (Sterpetti, Cucina *et al*. 1993; Fitzgerald, Shepherd *et al*. 2008). FSS was found to have to have a remarkable impact on gene expression in comparison with cells cultured under static conditions. Among a large number of genes which were differentially regulated by FSS tissue factor pathway inhibitor 2 (TFPI-2) and Bmp-2 were strongly overexpressed whereas endothelin-1, cadherin 11 and elastin were most downregulated. Comparing expression of cell specific genes, we found that expression of markers specific for EC was similar to static controls whereas few SMC differentiation markers were affected by FSS indicating that hemodynamic conditions do not induce significant changes in cell phenotype within the first 24 hours of exposure to FSS.

Using real-time PCR and Western Blot, we could confirm a major increase in the expression of TFPI-2 in response to FSS. This protein has previously been shown to suppress proliferation of ECs, activate SMCs and have anticoagulant properties (Petersen, Sprecher *et al*. 1996; Shinoda, Yui *et al*. 1999; Xu, Maiti *et al*. 2006).

In order to verify the effect of FSS on TFPI-2 expression *in vivo* we utilized the rat carotid balloon injury model. We found that TFPI-2 was expressed abluminally in the neointima 10 days after the injury in contrast to intact arteries where TFPI-2 was not detected. In this model, the endothelial-denuded luminal surface is covered by platelets for the first 24 hours after injury. As SMCs migrate across the IEL and proliferate, a lining of SMCs cover the luminal surface and become exposed to FSS. This leads to a resolution of the luminal thrombosis and a reconstitution of a non-thrombogenic surface by SMCs. It has been suggested that SMC surface heparan sulfates and induced

expression of iNOS may be involved in this process (Yan, Yokota *et al*. 1996). The physiological role of an increased TFPI-2 expression by SMCs in response to FSS requires further investigation. Possibly, SMC expressed TFPI-2 may contribute to the non-thrombogenic condition of the intimal surface in the absence of ECs. It may also suppress thrombosis by interfering with the tissue factor pathway.

Staining for von Willebrand factor confirmed the absence of an endothelial layer in the injured vessels. However we observed a diffuse abluminal staining for von Willebrand factor in the injured carotid artery. In a previous report where p53 null mouse SMCs exposed to FSS were shown to trans-differentiate to a EC-like phenotype (Wang, Yan *et al*. 2006). It is likely that endothelial cell markers may be expressed at later time periods since there was no change in genes specific for ECs after 24 hours of FSS exposure. Moreover, neointimal SMC are exposed to both mechanical and chemical stimuli in the rat model but only to FSS during *in vitro* conditions.

DNA synthesis was analysed in cultured rat SMCs and human ECc in response to treatment with recombinant human TFPI-2 (rhTFPI-2) in order to determine if TFPI-2 was involved in the antiproliferative effect of FSS. In accordance with a previous report, TFPI-2 inhibited human saphenous vein EC proliferation (Xu, Maiti *et al*. 2006). Inhibition of EC proliferation by TFPI-2 may take place after iatrogenic vascular injury such as endarterectomy or balloon angioplasty where the vascular luminal surface is denuded, and SMCs as well as sub-intimal structures become exposed to FSS. In the rat carotid injury model, regeneration of ECs begins early after injury but stops 6 weeks following injury, and is limited so that the middle segment of the vessel remains without an EC lining (Kohler and Jawien 1992). It is possible that complete regeneration of the endothelium in the rat model is prevented by TFPI-2, despite the counterintuitive association between EC denudation, induction of TFPI-2 in SMCs in response to FSS, and the ability of TFPI-2 to inhibit EC proliferation.

Proliferation of SMCs was also decreased by rhTFPI-2 but at much higher, probably supraphysiological, concentrations. This observation is in contrast with the mitogenic effect of TFPI-2 on SMCs reported previously (Shinoda, Yui *et al*. 1999). Our findings suggest therefore that TFPI-2 preferentially inhibits EC proliferation after vascular injury, whereas the inhibition of SMC proliferation is more uncertain.

In this study we demonstrate that increased levels of FSS downregulate SMC proliferation and significantly alters gene expression. TFPI-2 is strongly upregulated by FSS in SMCs and has an ability to inhibit proliferation of both ECs and SMCs which suggest a role for this molecule in the regulation of EC regeneration and influence

luminal thrombosis. These results imply that hemodynamic forces can directly effect SMC gene expression and in this way regulate intimal repair.

4 CONCLUDING REMARKS

To summarize the work presented in this thesis, where the focus has been directed at repair processes in the vessel wall, I would like to emphasize some of our findings.

IGF-1 signalling is essential in vascular development and other systems but is not expressed in the mature vessel wall under physiological conditions. However, the IGF-1 axis is actively driving healing processes in situations like atherosclerosis, neoangiogenesis and, particularly, in restenosis. In contrast to other growth factors, studies on the role of the IGF-1 system as a target for inhibition of intimal hyperplasia has been limited by the absence of specific pharmacological agents that do not interfere with insulin signalling. With the development of picropodophyllin, which specifically attenuates IGF-1R phosphorylation such investigations has become possible. Our results imply that PPP can be used to inhibit extensive healing reactions in the vessel wall such as restenosis in humans. However, since surgical interventions are rarely performed on normal vessels, it is important to remember that IGF-1 signaling may also be central for repair processes in atherosclerotic lesion by regulating SMC function, fibrous cap formation, and thus in the end IGF-1 may be crucial for plaque stability. At the same time, the IGF-1 pathway plays a key role in neoangiogenesis, which can contribute to intraplaque hemorrage, a feature associated with plaque progression and rupture. Thus, the role of the IGF-1 system in development, progression and stabilisation of the atheroma requires further studies. We are currently trying to dissect these mechanisms by inhibiting IGF-1R signalling using oral feeding of PPP in the ApoE null mice model of atherosclerosis. As a possible drug for prevention of restenosis, PPP should most likely be administrated using local delivery platforms such as in DES or local perioperative application for open surgical procedures (Nugent, Groothuis et al. 2002; Matyas, Berry et al. 2008). PPP has been shown to suppress tumor progression in mice by inhibition of neovascularisation and cell migration. These effects may partly be mediated through regulation of cell matrix interactions by production of matrix metalloproteinases *in vitro* (Haga, Yamashita *et al*. 2003). This suggests another possible role for the IGF-1 system in vascular disease where MMP activity is an important pathological component. PPP was developed as an antitumor compound and the first clinical studies on patients with cancer are being conducted. Such treatment may also affect the cardiovascular system of the study subjects, which must be evaluated for relevant concentrations and conditions.

Besides humoral factors, hemodynamic forces play an important role in regulation of vessel wall homeostasis and adaptation to physiological and pathological conditions. This is largely realized through the ability of ECs to sense fluid shear stress and to transfer it in to signalling cascades that affect SMC proliferation and survival, inflammation and lipid metabolism in the vessel wall. However, in some situations even SMCs are exposed to blood flow and FSS. In paper IV exposure to FSS inhibited proliferation of SMCs and considerably altered the expression of many genes. Some of these genes may be involved in the antiproliferative effect of FSS. Thus TFPI-2, which was markedly overexpressed by FSS, inhibited proliferation of ECs and SMCs *in vitro*. Secondary SMCs stimulated with serum may represent activated SMCs deep in the intima after injury while cells exposed to FSS in culture may correspond to SMCs which are present migrated to the luminal layers of the intima. Indeed, positive staining for TFPI-2 was found in abluminal SMCs of the rat neonitima. It would be of interest to investigate if TFPI-2 can attenuate SMC proliferation induced by low and disturbed flow *in vivo* or oscillatory shear stress *in vitro* (Haga, Yamashita *et al*. 2003). Additionally, targeting TFPI-2 signaling would allow to study the mechanisms of SMC regulation by flow. Similar to IGF-1, TFPI-2 has also been shown to be involved in cancer. It can suppress tumor invasion, growth, metastasis and angiogenesis through regulation of MMP activity and through direct inhibition of the tissue factor pathway. Regulation of healing reactions in the vessel wall by FSS may involve the same mechanisms and should be further investigated.

In papers I and IV, we utilized the rat carotid injury model, which, like other small animal models, has many advantages when studying mechanisms of vessel wall healing or to test new pharmacological agents as discussed above. Ultrasound biomicroscopy, a helpful tool for such studies, was evaluated in paper II. It was found to be reliable method to determine blood flow, vessel diameter, structural changes in both the intima and media, and thus to follow the temporal fashion of vascular repair *in vivo*. As histological microscopy, UBM does not end at the level of structural visualisation, but is also useful for molecular imaging in living animals. Importantly, ultrasound visualisation techniques provide a platform for translational studies in the field of vascular biology since ultrasound imaging is widely used for both routine examinations and clinical studies of human vasculature. In paper III, we also found that ultrasound morphology can characterize the phenotype of human carotid atherosclerotic lesions. High carotid plaque echodensity positively correlated with expression of the IGF-1 gene, which supports our hypothesis that similar repair mechanisms may underlie

stabilisation of atherosclerotic lesions as the intimal response to injury. Plaques obtained from asymptomatic patients, individuals treated with statins and lesions which recently caused thromboembolism were characterized by higher levels of mRNA coding for SMC specific genes or ECM components while expression of matrix degrading proteases and inflammatory cytokines was lower in these lesions. The classification of genes used in paper III, especially the separation of inflammation and genes encoding healing, or fibrotic, processes may appear artificial. However, some approximation or simplification is necessary when global gene expression analysis is performed. We therefore propose the use of our described clinical variables when searching for unknown mechanisms of plaque stabilisation and to identify new target molecules for pharmacological promotion of plaque healing.

Even though we mainly focused on the role of SMCs in vessel repair after vascular injury and in plaque instability, one must remember that. healing in the vessel wall also takes place in other conditions and involve many other cell types with important functions. Nevertheless, the central role of SMCs can not be neglected and I therefore believe that our findings will contribute to the understanding of vascular repair mechanisms and provide grounds for new strategies to improve outcome for patients with cardiovascular disease.

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