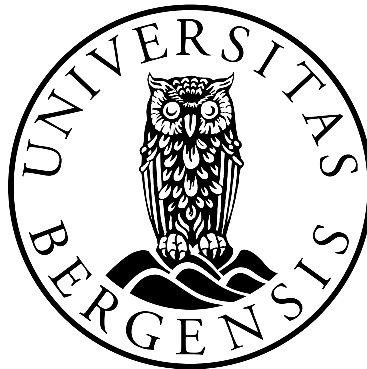
Intimal Hyperplasia

Experimental and Clinical Studies

Vegard Skalstad Ellensen



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Paper I

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Scientific Environment

All work in Papers I-III was performed under the guidance of Professor Torbjørn Jonung and Dr. Gustav Pedersen at the Department of Vascular Surgery, Haukeland University Hospital and the Department of Surgical Sciences and Bergen, University of Bergen. All work related to Paper I was performed at the Vivarium, Haukeland University Hospital. Work relating to Papers II and III were performed in collaboration with the Department of Biomedicine, University of Bergen, under direction of Professor Jim Lorens. Paper IV was performed at the Section of Cardiothoracic Surgery, Department of Heart Diseases, Haukeland University Hospital, under direction of Professor Rune Haaverstad.

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Abstract

Introduction

Intimal hyperplasia (IH) is recognized as the primary cause of reocclusion 2-24 months following vascular intervention and surgery, reportedly occurring in 30-50% of patients. It is an exaggerated form of the normal healing process of the iatrogenic trauma. The two main processes of IH are proliferation and migration of vascular smooth muscle cells (VSMCs) with a subsequent deposition of extracellular matrix. This accumulation of cells and matrix leads to a narrowing or, ultimately, occlusion of the lumen. IH usually affects arterial anastomoses, but a very distinct type of IH called pannus also exists. It occurs at the valvular annuli of the heart following implantation of valve prostheses and can obstruct valvular motion, thereby leading to acute heart failure. The treatment for IH is reintervention, resulting in major clinical issues for the patients and strain on the health care system. No prophylactic treatment currently exists. Heparins have demonstrated inhibitory effects in cell and animal experiments, but the results of clinical studies have been disappointing. There are no known predisposing factors enabling one to foresee who will develop clinically significant IH and who will not.

This thesis sought to investigate the processes of IH in cell cultures, the effects of different heparins on these processes and the clinical manifestations in animals and patients.

Paper I

The aim of this study was to investigate if heparin coated vascular grafts could improve patency and recruit less IH compared to uncoated grafts at a longer term in a sheep model. Twenty-eight common carotid arteries in fourteen sheep were bypassed with a luminal heparin-coated graft on one side and a control uncoated contralaterally. The grafts were explanted after six months. The thickness of IH in open grafts was measured with histomorphometrical methods.

In conclusion, heparin coated grafts improved patency and recruited less IH compared to control uncoated grafts at six months in a sheep model.

Paper II

This study was designed to investigate the influence of heparin on the processes of IH of patient-derived vascular cell mono- and co-cultures. VSMCs and endothelial cells (ECs) were isolated from vessel wall biopsies from six patients. The cell cultures were treated with heparin and evaluated for effects on proliferation, migration and mitogen-activated protein kinase - extracellular signal-regulated kinase (MAPK-ERK) signal transduction using image-based cell enumeration, real-time migration monitoring and flow cytometry.

Heparin inhibited proliferation and migration of patient-derived VSMC cultures, but only at concentrations exceeding clinical doses. The proliferative response of VSMCs to heparin was not affected by the presence of ECs. Heparin stimulated MAPK-ERK phosphorylation of VSMCs at lower concentrations in the presence of human fibroblast growth factor (hFGF). MAPK-ERK phosphorylation of unstimulated cultures was inhibited dose-dependently. Thus, heparin seems to influence patient-derived vascular cells through both a stimulatory hFGF-dependent and an inhibitory hFGF-independent pathway. These findings may explain the divergence of results between previous *in vitro* and clinical studies and provide a basis for new therapeutic strategies.

Paper III

This study was designed to investigate the effects of two low-molecular weight heparins (LMWHs), enoxaparin and dalteparin, compared to unfractioned heparin on proliferation, migration and MAPK-ERK signal transduction of patient-derived VSMCs. VSMCs were treated with the LMWHs in a range of concentrations and evaluated using image based cell enumeration, real time migration monitoring and flow cytometry. Series treated with unfractioned heparin were included as positive controls and untreated series as negative controls.

This study demonstrated a difference in proliferative and migratory effects between the two LMWHs and unfractioned heparin in patient-derived VSMCs. The effects corresponded to the MAPK-ERK phosphorylation, suggesting different

mechanisms of action. These results may explain why clinical trials using LMWHs to prevent IH have failed to observe a reduced incidence of restenosis and do not support prolonged therapeutic use to prevent it.

Paper IV

The aim of this study was to analyse the incidence, treatment and results of acute prosthetic valve obstruction due to IH overgrowth originating from the periannular area (pannus), in a large series of patients with a Medtronic-Hall tilting disc aortic prosthesis. This was a retrospective study of patients seen at Haukeland University Hospital over the course of the last 30 years. Special attention was given potential predisposing factors of pannus development.

This study demonstrated that females and younger patients are at higher risk for pannus development. When acute dysfunction of a mechanical aortic valve by pannus is suspected, an immediate echocardiogram and an emergency aortic valve replacement should be performed to prevent an otherwise fatal outcome.

List of Publications

I. **Improved Patency and Reduced Intimal Hyperplasia in PTFE Grafts with Luminal Immobilized Heparin Compared with Standard PTFE Grafts at Six Months in a Sheep Model.**
Pedersen G, Laxdal E, **Ellensen VS**, Jonung T, Mattsson E
J Cardiovasc Surg (Torino), 2010;51(3):443–448.

II. **Heparin Does Not Inhibit Intimal Hyperplasia Processes on Human Vascular Cells at Clinical Concentrations due to Antagonistic Pathways.**
Ellensen VS, Abrahamsen I, Lorens J, Jonung T
Submitted.

III. **Effects of Enoxaparin and Dalteparin on Proliferation and Migration of Patient-Derived Vascular Smooth Muscle Cells**
Ellensen VS, Abrahamsen I, Lorens J, Jonung T
Accepted 26.11.2013, VASA - European Journal of Vascular Medicine.

IV. **Acute Obstruction by Pannus in Patients With Aortic Medtronic-Hall Valves: 30 Years of Experience.**
Ellensen VS, Andersen KS, Vitale N, Davidsen ES, Segadal L, Haaverstad R
Ann Thorac Surg, 2013;96:2123-2128.

List of Abbreviations

AKT	protein kinase B
ANOVA	analysis of variance
AT	antithrombin
ATIII	antithrombin-III
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CBAS	Carmeda BioActive Surface®
CLI	critical limb ischemia
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylene-diamine-tetraacetic acid
EGM-2	endothelial cell growth medium-2
ePTFE	expanded polytetrafluoroethylene
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GAG	glucosaminoglycan
H-E	haematoxylin-eosin
hFGF	human fibroblast growth factor
HSPG	heparan sulfate proteoglycan
IC50	half maximal inhibitory concentration
ICAM-2	intercellular adhesion molecule 2
IGF	insulin-like growth factor
IH	intimal hyperplasia
LMWH	low-molecular weight heparin
MAPK-ERK	mitogen-activated protein kinase - extracellular signal-regulated kinase
MEK1	mitogen activated protein kinase kinase
MMP	matrix metalloproteinase

MPK-1	mitogen-activated protein kinase-1
MT-MMP-1	membrane bound matrix metalloproteinase-1
NO	nitrous oxide
OR	odds ratio
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGF-BB	platelet-derived growth factor-BB
PDGFR	platelet-derived growth factor receptor
PFA	paraformaldehyde
PKC	protein kinase C
PTFE	polytetrafluoroethylene
SmBM-2	smooth muscle basal medium-2
SmGM-2	smooth muscle growth medium-2
SNAC	N-[8-(2-hydroxybenzoyl)amino]caprylate
t½	half-life
TGF-β	transforming growth factor-β
TRITC	tetramethyl-rhodamine isothiocyanate
UEA-I	*Ulex europaeus* agglutinin I
UH	unfractioned heparin
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

Introduction

Intimal hyperplasia (IH) is recognized as the primary cause of reocclusion 2-24 months following vascular intervention and surgery, reportedly occurring in 30-50% of patients[1-3]. It is an exaggerated form of the normal healing of the iatrogenic trauma. The two main processes of IH are proliferation and migration of vascular smooth muscle cells with a subsequent deposition of extracellular matrix[4,5]. This accumulation of cells and matrix leads to a narrowing or, ultimately, occlusion of the lumen (Fig. 1). IH usually affects arterial anastomoses, but a very distinct type of IH called pannus also exists[6]. It occurs at the valvular annuli of the heart following implantation of valve prostheses and can obstruct valvular motion, thereby leading to acute heart failure.

The treatment for IH is reintervention, resulting in major clinical issues for the patients and strain on the health care system. There are no known predisposing factors enabling one to foresee who will develop clinically significant IH and who will not. Despite extensive investigation since the first report of IH by Dr. Carrell in 1906, an effective prophylactic treatment has not been found[7]. Heparins have demonstrated inhibition of the IH processes in animal models[8-24]. However, the results from treatment of IH with heparins in patients have been disappointing[25-31]. This discrepancy of results may be due to several factors, but diverging experimental conditions are the most conspicuous. Drug eluting stents with a range of compounds and different grafts have also been investigated, none with convincing effects on the formation of IH.

This thesis sought to investigate the processes of IH in cell cultures, the effects of different heparins on these processes and the clinical manifestations in animals and in patients.

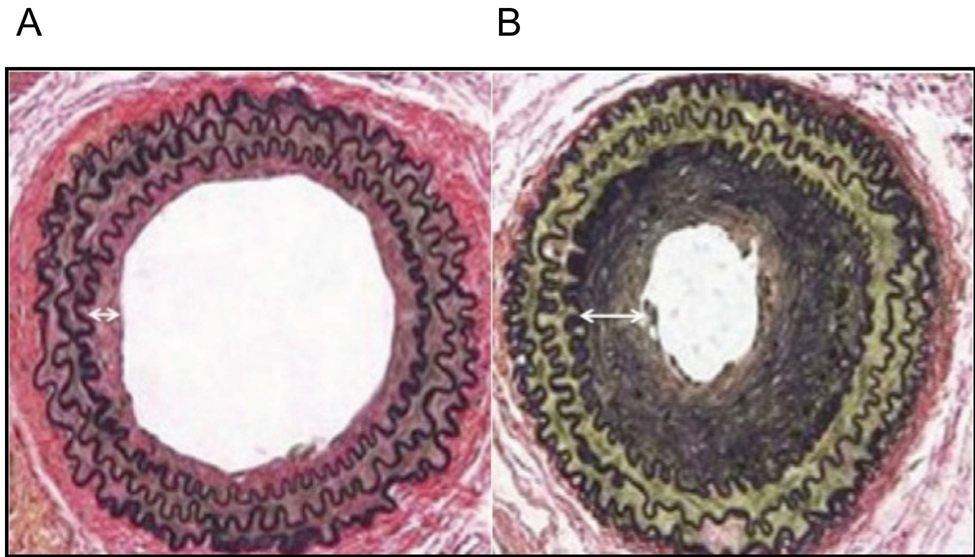

Figure 1. Intimal hyperplasia.

A: *Normal tunica intima (white arrow) of an artery.* **B:** *Thickened tunica intima due to intimal hyperplasia (white arrow) of an artery.*

1. Background

1.1 Arterial Anatomy

The vascular system is mainly derived from the splanchnic mesoderm layer, but vascular smooth muscle cells develop from several embryological distinct origins[32,33]. It appears in the middle of the 3rd week of the embryonic development in response to insufficient nutritional supply by diffusion alone[34]. The further angiogenesis results in a common structure of arteries. An artery consists of three main layers, or tunics (L. *tunica,* coat): tunica intima, media and adventitia (Fig. 2).

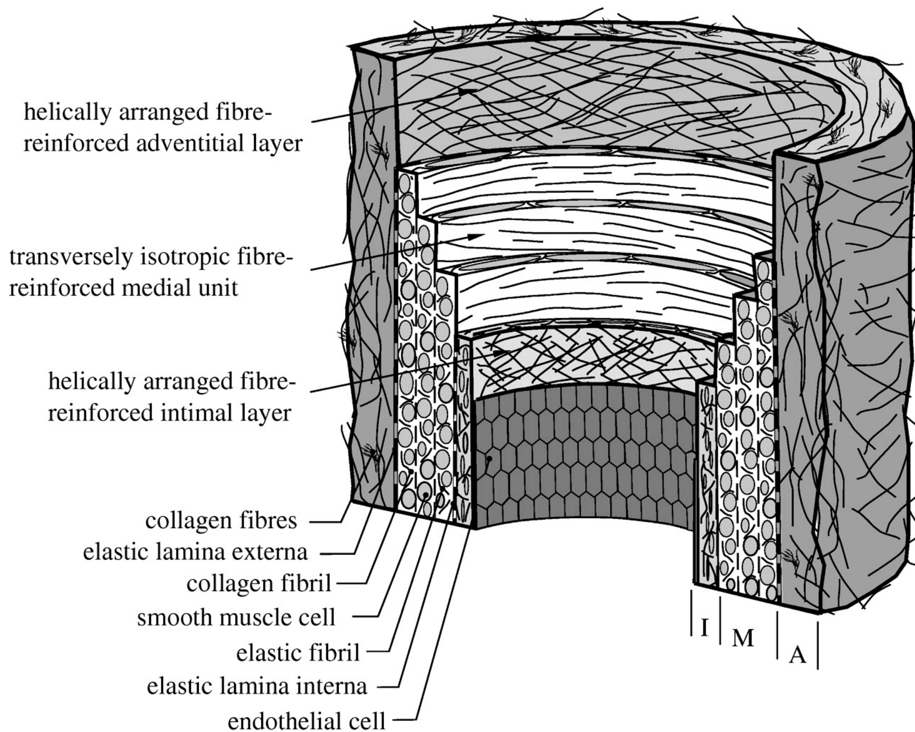

Figure 2. Layers of the arterial wall.

I=intima; M=media; A=adventitia.

Reproduced with permission, Gasser et al., J. R. Soc. Interface 2006;3:15-35, Fig. 1.

1.1.1 Tunica Intima[35,36]

The intima has four layers. Innermost is a monolayer of flattened endothelial cells (ECs) linked by occluding junctions that prevent leakage of even small molecules. This lining is in direct contact with blood and must thus exhibit antithrombotic properties. These endothelial cells rest on a basal lamina. Underneath the lamina lies the subendothelial layer. It consists of loose connective tissue and, in larger arteries, scattered vascular smooth muscle cells (VSMCs) in a longitudinal orientation. An internal elastic membrane separates the subendothelial layer, and thus the intima, from tunica media. It is composed of elastin and forms a fenestrated sheet to allow for diffusion of substances and nutrients to the deeper parts of the vessel wall. The endothelium has processes that pass through the fenestrations and make gap junctions with VSMCs in tunica media, thus allowing for signal transmission.

1.1.2 Tunica Media[35,36]

This layer consists mainly of VSMCs concentrically arranged helically in layers transversely to the long-axis of the vessel. Each muscle cell is enveloped in a basal lamina. To communicate with the other muscle cells, ensuring a coordinated vasomotor tone, processes extend through intervals in the basal lamina creating gap junctions. Variable amounts of elastic lamellae, reticular fibers (collagen type III) and proteoglycans are interspersed in between the muscle cells. In larger arteries an external elastic lamina is found, separating media from adventitia.

1.1.3 Tunica Adventitia[35,36]

Adventitia is largely composed of longitudinally oriented collagen type I, elastic fibers and fibroblasts, but also harbours vasa vasorum, lymphatic capillaries and vasomotor nerves. The adventitia eventually blends with the surrounding connective tissue of the surrounding organ.

1.2 Endothelial Cells

Endothelial cells (ECs) are found in the tunica intima and constitute the epithelium lining the vascular system, from the heart to the capillaries. They contribute to several

functions necessary for the maintenance of life: barrier function and fluid filtration, hemostasis and thrombolysis, regulation of vasomotor tone, inflammation and angiogenesis. ECs respond respond to both physical (blood flow dynamics) and chemical cues, and elaborate growth regulators/-factors and vasoactive substances like glucosaminoglycans (GAGs), prostacyclin, nitrous oxide (NO) and endothelins[11,37-39].

1.3 Vascular Smooth Muscle Cells

Vascular Smooth Muscle Cells (VSMCs) reside mainly in the tunica media, but may be present in the subendothelial layer of tunica intima (pericytes). They constitute the contractile element in vessels and thereby the regulatory apparatus of vascular tone. Under normal circumstances VSMCs have a low proliferation and apoptosis rate[40,41]. Increased turnover is thought to be involved in atherosclerosis and IH[40-45]. Even though VSMCs are considered one type of cell, this is actually a heterogeneous group of different phenotypes. Whether this is determined by differences in embryological origin (Section 1.1), biochemical influence or both has not been settled. However, there is evidence that at least two main phenotypes are involved in IH: the contractile differentiated and the synthetic dedifferentiated[32]. The contractile differentiated type has a low rate of proliferation and exhibit contractile properties. This phenotype is induced by, among others, insulin-like growth factor (IGF), heparin and transforming growth factor-β (TGF-β). The synthetic dedifferentiated phenotype is induced by basic fibroblast growth factor (bFGF) and platelet-derived growth factor-BB (PDGF-BB) and characterised by a high rate of proliferation, migration and protein synthesis. Phenotypic switching is controlled mainly via the protein kinase B (AKT), protein kinase C (PKC) and mitogen-activated protein kinase - extracellular signal-regulated kinase (MAPK-ERK) pathways and may occur at any time given the right circumstances (Fig. 3)[46].

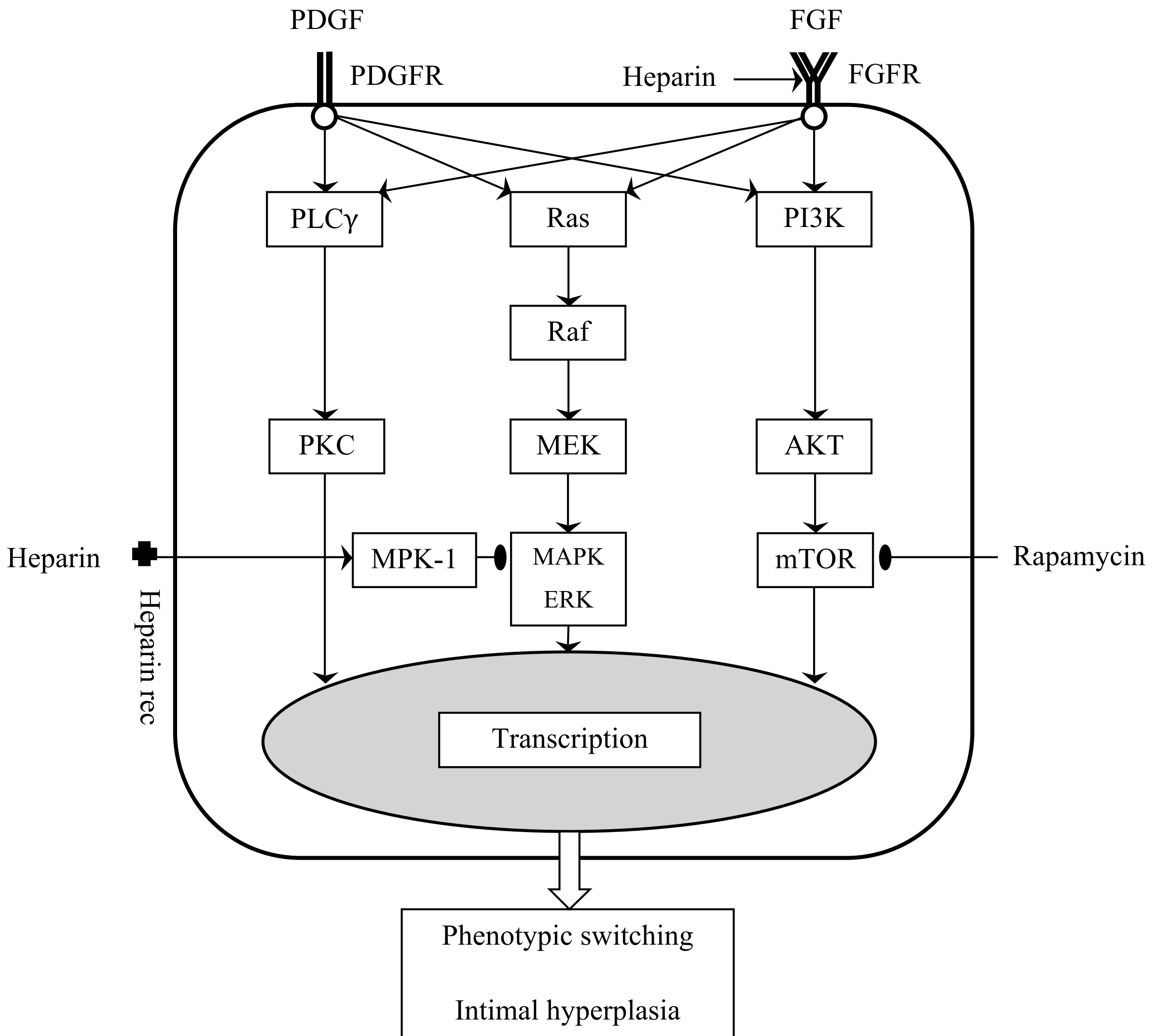

Figure 3. The main pathways of phenotypic switching and IH.

Arrows indicate stimulatory signals and the clubbed lines indicate inhibitory signals. See List of Abbreviations for details. Modified from Muto et al., J Vasc Surg 2007;45:15A-24A.

1.4 Intimal Hyperplasia

Synonyms: myointimal/neointimal hyperplasia/thickening

Intimal hyperplasia (IH) is abnormal migration and proliferation of VSMCs with subsequent deposition of extracellular connective tissue matrix in response to damage. It is an exaggeration of the normal healing process. Iatrogenic injury occurs whenever vascular intervention or vascular surgery is performed. It is not possible to foresee who will develop an exaggerated reaction and who will not. The abnormal healing reaction leads to a narrowing and, ultimately, occlusion of the vascular lumen (Fig. 1). The type of injury is classified as[47]:

Type I	Functional alterations without significant morphologic change
Type II	Endothelial denudation without intimal and medial damage
Type III	Endothelial denudation with intimal and medial damage

Angioplasty and arterial endarterectomy both result in type III injury, while venous grafts exhibit generalized type I and II injury in addition to type III at the anastomosis.

Injury triggers the complex response of IH, involving several biochemical pathways and cascades resulting in the two predominating processes of IH: proliferation and migration. Accumulation of synthetic dedifferentiated VSMCs in the intima leads to a third important process, synthesis of extracellular matrix (ECM). The processes take part in different temporal phases of IH-formation, as described in Section *1.4.4*.

1.4.1 Proliferation

As described in Section 1.3 and as illustrated in Figure 2, PDGF and bFGF stimulate phenotype switching to the synthetic dedifferentiated type and thus a proliferative response in medial VSMCs. bFGF is released from damaged ECs and VSMCs, while PDGF is mainly secreted from activated platelets and to some extent from endothelial and vascular smooth muscle cells. Under normal circumstances medial VSMCs have a low turnover rate of approximately 1 %. After balloon angioplasty, this rises to 20

% after 48 hours[48-50]. This represents the first proliferative phase. The fraction of cells in mitosis reaches its maximum between three and seven days. VSMCs start migrating into the intima at this point. The medial proliferation response returns to baseline after four weeks. In the intima approximately 50 % of the migrated VSMCs will proliferate further[5]. This second proliferative phase reaches a maximum 14 days after reaching the intima, before returning to baseline after 4 weeks. However, it may be prolonged especially in areas where the re-endothelialisation is slow. The second proliferative phase is controlled by re-endothelialisation, para- and autocrine factors.

1.4.2 Migration

Migration occurs after the initial proliferative phase of VSMCs 3-7 days after injury. This process is partly mediated by PDGF. As mentioned previously, VSMCs are individually enveloped in a basal lamina within tunica media (Section *1.1.2*). To be able to migrate on command given by PDGF and bFGF, this lamina must be demolished. There is evidence that PDGF and bFGF stimulates this process by inducing secretion of proteases from the VSMCs themselves[39]. The secreted proteolytic enzymes accumulate at the leading edge of migration. These enzymes are mainly the matrix metalloproteinases (MMPs) and enzymes from the plasmin system. Plasmin also activates the membrane bound matrix metalloproteinase-1 (MT-MMP-1), leading to further amplification of migration. Up to 30% of medial VSMCs may migrate in response to injury and, reaching intima, approximately 50% of the VSMCs undergo further mitosis[5].

1.4.3 Extracellular Matrix - ECM

The ECM production is most prominent in the chronic phase of IH. It may constitute up to 80 % of IH substance[51]. Collagen, elastin and proteoglycans are synthesized and deposited by the dedifferentiated, proliferated and migrated intimal VSMCs.

1.4.4 Phases of IH

The processes of IH proceed simultaneously, but in an orderly temporal fashion (Sections *1.4.1-3*). Three different phases are recognized - the *hyperacute*, *acute* and the *chronic*[5,47]:

1.4.4.1 Hyperacute Phase

In the immediate time following vascular injury, platelets adhere to the exposed collagen, laminin and von Willebrands factor in the wall via integrins. Binding to collagen activates the platelets enabling them to change conformation, discharge granules and stick to other platelets. PDGF is the main substance secreted from the platelets, regulating proliferation and migration. Damaged VSMCs release both some PDGF and larger amounts of bFGF. These substances induce VSMC phenotype switching from the low proliferative contractile differentiated to the synthetic dedifferentiated type with a high rate of proliferation, migration and protein synthesis.

1.4.4.2 Acute Phase

Within 24 hours, VSMCs start proliferating in the media as a response to the elevated levels of bFGF. After approximately four days migration of VSMCs from the media, through the fenestrations of the internal elastic lamina, to the intima can be observed. Reaching the intima, approximately 50 % of the migrated VSMCs will proliferate further, while some undergo apoptosis. This balanced and constantly enduring process is regulated by the extent of damage to the cells and the local microenvironment. Proliferation of VSMCs in the intima is downregulated when the endothelial monolayer has been regenerated. It is not known which mechanisms are responsible, but the endothelium may act by re-establishing the barrier, secretion of inhibitory substances or both. This phase lasts up to 12 weeks.

1.4.4.3 Chronic Phase

The chronic phase follows the acute and is distinguished by VSMC synthesis of ECM consisting of collagen, elastin and proteoglycans. The synthesis is regulated by

cytokines and growth factors. There is a constant remodelling regulated by the MMP- and plasminogen-plasmin systems i.e. IH is a dynamic process.

1.5 Signalling

1.5.1 Signalling Pathways

Several pathways are involved in the formation of IH, but the most important ones, mediated by FGF and PDGF, are MAPK-ERK, AKT and PLC-γ/PKC (Fig. 3). The resulting effects depend on degree of stimulation, inhibition and modulation. The most important with regard to IH is the MAPK-ERK pathway. It is involved in regulation of a variety of cellular functions such as gene expression, mitosis, proliferation, differentiation and apoptosis[52-54]. Of particular interest is the inhibitory action of mitogen-activated protein kinase-1 (MPK-1) on MAPK-ERK as it seems to be controlled by heparin receptor-heparin interaction[55]. The heparin receptor has yet to be fully characterized.

1.5.2 Heparin

Heparin is a highly sulfated GAG with the highest negative charge density of all known biological molecules[56]. Its biologic half-life (t½) is between 23 minutes to 2.48 hours[57]. It is closely related in both structure and action to heparan sulfate proteoglycan (HSPG) present *in vivo*. Commercially available heparin is of porcine or bovine origin, with an average molecular weight of 12-15 kDa[58]. The molecular structure is based on repeating disaccharide units (IdoA-GlcNS most common) with varying degree of sulfatation. This results in a helical structure with clusters of sulfate groups on either side. It is used in clinical practice mainly for its anti-coagulative properties. Full clinical heparinization is achieved at serum concentrations of 2-7 μg/mL (Felleskatalogen AS, Norway). However, heparins have been shown to inhibit both VSMC proliferation and migration, and thus the formation of IH[8-11,14-16,59]. The anti-proliferative effect has been reported in both animal and human vascular cells. There is evidence that the inhibitory effect is correlated to the number of GAG-groups and their degree of sulfatation[60,61]. Heparin is thought to exert its inhibition on the proliferation and migration of VSMCs by binding to a heparin receptor and

thereby inhibiting the MAPK-ERK pathway via MPK-1 (Fig. 3)[55]. Heparin is also vital in the dimerization of FGF-receptors, increasing their affinity for FGF and subsequently the stimulation of the MAPK-ERK pathway (Fig. 3).

1.5.3 Low-Molecular Weight Heparins - LMWHs

Low-molecular weight heparins (LMWHs) are synthesized by producer-specific GAG-chain cleavage of unfractioned heparin (UH), removing the thrombin-binding sequence in proximity to the pentasaccharide antithrombin (AT)-site. This results in preservation of the anticoagulative properties and a shortened GAG-sequence. Different LMWHs exhibit dissimilar physical, chemical and biological properties. They are defined as heparin salts with an average weight of below 8000 Da, and at least 60% of the molecules need to weight less than 8000 Da. Enoxaparin (Klexane®, product details from Sanofi-Aventis, Paris, France) is one LMWH, derived from the intestinal mucosa of pigs. Its average molecular weight is 4500 Da. Dalteparin (Fragmin®, product details from Pfizer, New York, NY, USA) is another widely used LMWH, also derived from pigs' intestinal mucosa. Its average molecular weight is 5000 Da. Due to ease of administration and more predictable effects, LMWHs are preferred to UH in clinical practice and often considered as synonomous drugs.

1.6 Pannus

Pannus is the designation of IH at its most proximal occurrence – the valvular annuli of the heart. Its formation is due to the response of the periannulus and becomes clinically evident months or years after implantation of valve prostheses. Even if the processes of IH seem to be theoretically restricted to the first year following intervention, histopathologic examination of pannus surgically removed several years following valve implantation have revealed IH and not atherosclerosis[6]. One explanation may be that the valve *per se* and the shear stress from the blood flow cause a constant mechanical trauma with subsequent maintenance of the IH-formation. When pannus growth is limited to the periannular area, the patient may remain asymptomatic. With continued growth, however, pannus may interpose between the prosthetic valve ring and disc (Fig. 4). Hence, it may inhibit opening or

closure of the valve disc, thereby leading to acute aortic obstruction or regurgitation with a potential of fatal outcome.

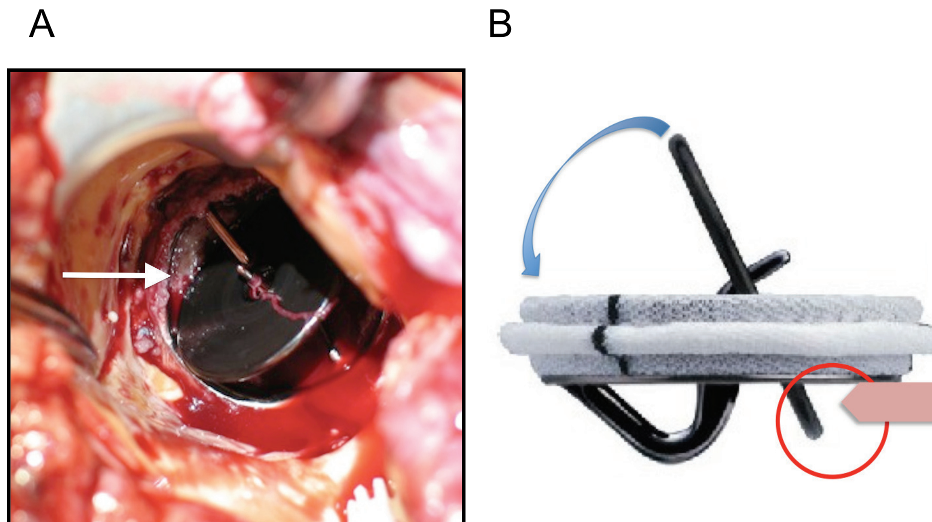

Figure 4. Localization of pannus in obstructed Medtronic-Hall aortic valve prostheses.
A: *The valve disc is blocked in an open position due to pannus (white arrow) in the minor valve orifice.* **B:** *Schematic view of pannus (pink arrow) obstructing the valve disc in the minor orifice (red circle), blocking the normal motion (blue arrow).*

1.7 Prevention of IH Using Heparins: a Historical Perspective

Alexis Carrel was the first to report IH in 1906[7]. He described the occurence of a glistening substance covering the stiches placed in making the anastomosis within a few days after the operation. Though, it was not until the mid 1970s that a thorough investigation of this pathology and its pathogenesis commenced. During the last 40 years, substantial effort has been made to find a way of treating IH.

1.7.1 Laboratory Studies

Pioneering work was done by Morris J. Karnovskys group, who developed a rat carotid model to study endothelial regeneration and the effects of endothelial

denudation in large arteries[62]. Parallel studies by other groups on rabbits and baboons treated with dipyridamole and anti-platelet serum reported decreased myointimal thickening[63,64]. Through a series of studies using the carotid rat model, Karnovskys group created a base of knowledge about neointimal formation and hyperplasia. In one of their first papers, heparin was suggested and tested as a possible inhibitor of myointimal thickening[8]. Their conclusion was that heparin in doses sufficient to prolong clotting markedly suppressed intimal VSMC proliferation after arterial endothelial injury. This initiated a new era of research on IH. Other substances with antiplatelet activity, e.g. aspirine, reserpine and flurbiprofen, were tested, but without effect on myointimal thickening[65].

In 1980, Rosenberg of Karnovskys group discovered that heparin could be separated into anticoagulant and non-anticoagulant fractions. These two fractions were given to rats after balloon denudation using the rat carotid model[66]. There was significant inhibition of myointimal growth that correlated with total mass of heparin administered, regardless of anticoagulant activity. Hoover et al. came to the same conclusion using aortic cells from the same rat species[9]. Further, this study postulated that there was probably an interaction of heparin with the cell surface. This observation led to a study of the binding and internalization of radiolabeled and fluoresceinated heparin in VSMCs[67]. The findings of this study suggested that heparin binds to high-affinity surface-receptors and undergoes endocytosis. It was also emphasized that the internalized heparin concentrated in the perinuclear region. On the contrary, Majack et al. demonstrated that heparin causes the release of thrombospondin, an attachment protein, from the matrix into the media[68]. This finding raised the possibility that heparin affects SMC proliferation and migration via an extracellular mechanism, and without being bound and internalized.

During the first years of research on heparin and IH, the proliferative response was the prime focus. In 1984 Majack and Clowes demonstrated that heparin also possesses an anti-migratory effect in aortic rat cells, thereby inhibiting both main processes of IH[10]. This was further emphasized in a paper by Clowes et al. demonstrating that the inhibition of intimal thickening in injured rat artery by heparin is the consequence of both inhibition of VSMC entry into the growth fraction (G_1)

and migration of VSMCs from the media into the intima[69]. These effects were long lasting and not reversed even if the heparin was stopped after a short course of administration.

Edelman et al. highlights several challenges of using heparin clinically[70]. The half-life is short (1-5 hrs), therapeutic serum levels can only be maintained by continuous infusion and due to anticoagulative activity there is potential for bleeding complications. The former problem has been addressed by creating LMWHs with a longer half-life. Oral administration instead of continuous infusion is limited by inability of heparin to pass through the gastric mucosa intact. After degradation in the stomach however, the fragments pass the mucosa. Problem is that these fragments are tri- and tetramers, too short to affect proliferation and migration[71]. Oral heparin was nevertheless suggested as an alternative by utilizing N-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC) to facilitate gastric absorption[72]. However, this was an animal study and clinical trials have yet to be seen.

In 1992 two reports on the effects of heparin on IH were published[18,73]. Both groups used an animal model, minipigs and rabbits respectively, with a similar setup. However, their results were contradictory. Buchwald et al. reported excellent results, while Gimple et al. demonstrated no effect. The findings and why the results differed was discussed in an editorial comment[74]. It pointed out that different ways of drug administration, type of injury, types of heparin, animals and cholesterol level may be of influence. The first two factors, drug administration and type of injury, were thoroughly investigated by Rogers et al., who concluded that duration, dose, and site of delivery might explain why substances showing good results in the lab are unsuccessful clinically[75].

In his editorial comment, Clowes also emphasized that comparative studies with human and animal tissue are needed to determine if any of the animal models mimic the human response. On this basis, his group developed an angioplasty baboon model since they are genetically more closely related to humans[76]. Two groups of baboons received continuous infusion of either saline or the LMWH SR 80258A with an average molecular weight of 6000 Da. No significant difference between the two groups was detected. Of notice is that an LMWH was chosen instead of UH. They

reasoned this choice on previous inhibition of baboon VSMCs in culture by SR 80258A. Plasma concentrations of SR 80258A were 17.2±6.9 μg/mL and 10.4±2.7 μg/mL at seven and 28 days respectively.

Chan et al. proposed that the high molecular weight fractions of heparin possess higher antiproliferative activity[59]. At 100 and 1000 μg/mL there were significant inhibitory effects, but no inhibition of proliferation was seen at 10 μg/mL. On the contrary, in their figure 1, there seems to be a negative inhibitory effect at this concentration, but this was not commented on in the article. UH demonstrated significantly more pronounced inhibition than LMWHs. Of notice is that heparin and LMWHs from several different suppliers were used, then pooled and fractioned. The following year, the same group proposed decreased heparin sensitivity, in other words heparin resistance, as an explanation of why there was such interindividual discrepancy of results[12]. This was based on their finding of lower sensitivity to growth inhibition by heparin in vascular cells from patients with restenosis. The concept of heparin resistance was further investigated by Refson et al., who found correlation between decreased [^{3}H]-labeled heparin binding, responsiveness to heparin in human cell cultures and one-year outcome for infrainguinal vein grafts[14]. However, by using a concentration of 100 μg/mL heparin, direct clinical translation was impossible. They comment on this in their article and defend it by citing that Yla-Herrtuala et al. found similar concentrations of heparins in the extracellular matrix around the VSMCs of the vessel wall[77]. However, this article does not investigate the matrix concentration of heparin at a given serum concentration, but rather gives an overview of the composition and alterations of native GAGs in normal and pathologic coronary arteries.

A decade after Castellot and Majacks binding and signalling studies, Letourneur et al. published two papers complementing Castellots findings[78,79]. The uptake of [^{3}H]-heparin in heparin resistant and -sensitive VSMCs was compared. Both cell types had the same internalization, degradation and secretion kinetics initially, but it was rapidly (5-8 hrs) downregulated in the resistant cells, while upregulated in the sensitive. The affinity of heparin binding *per se* was not affected. Their conclusion was that heparin acts to upregulate its receptors and that was

required for the antiproliferative response. Another study using [^{3}H]-heparin further contributed to the understanding of intracellular actions of heparin[80]. The observation that [^{3}H]-heparin bound to a single class of binding sites in a saturable and reversible, time- and concentration-dependent manner, supported those of Refson et al. from the year before[14]. Further, their results suggested that no simple relationship between heparin receptor binding and the inhibition of DNA-synthesis exists. Internalization, intracellular metabolism and -degradation are possibly each modulating steps of the heparin action.

Several studies had defined the dose-response of heparin using animal cells in culture, but none had determined the serum concentration needed to achieve the tissue concentration necessary for inhibition of VSMCs. Using bovine cells and rats, Lovich and Edelman estimated the arterial concentration of heparin required to inhibit IH maximally after injury *in vivo* to be 0.3 mg/mL (300 µg/mL)[81]. This was approximately triple the concentration needed for a similar response in cell culture, and illustrates that the culture-results reflect the cellular response alone, while the dosing *in vivo* reflects a tissue response. Since these heparin concentrations would be incompatible with life, contemporary studies started focusing on clinically applicable concentrations of other substances and methods of administration.

Since LMWHs had shown encouraging results in cell culture and tempted with much longer t½ than UH and considerably easier administration (subcutanously twice daily), their properties were explored. In two sheep experiments, Ao et al. demonstrated significant inhibition of IH by UH and enoxaparin, both in high and low doses[13,19]. There was one control group and four treatment groups receiving either low- or high dose of either enoxaparin or dalteparin subcutaneously for four weeks. Both enoxaparin groups and low dose dalteparin were injected once daily, while high dosage dalteparin was injected twice daily. Both LMWHs reduced IH, dalteparin more efficiently as it was effective in low doses as well as high. Fletcher et al. did a similar experiment with similar results by implanting a Dacron patch into the common carotid artery of sheep[21].

Comment

Laboratory research provided evidence that heparin and its low molecular fractions reduce formation of IH by inhibition of proliferation and migration, thereby reducing the number of cells potentially producing matrix. With the exception of Chan and Refsons studies, all experiments used animal cells or were based on animal models. In the two studies using human cells, the cells were from the aorta and the saphenous vein. A range of doses, administration, duration of therapy, type of heparin/LMWH, species and type of injury have been used. With the exception of the carotid rat model developed by Karnovskys group, little consistency is seen. These studies do not report the dose-response of UH and LMWHs on the proliferative and migratory effects on human cells. The dose-response relationship of heparin in human cells has been described by Chan et al., but only at 10, 100 and 1000 µg/mL. As the clinical serum concentration of heparin at full anticoagulation is in the 2-7 µg/mL range (Felleskatalogen, Norway), their dose-response chart seems unsuited for evaluation of clinical applicability.

1.7.2 Clinical Studies

To test the edifying results from laboratory experiments, several clinical trials have been performed.

Ellis et al. administered heparin intravenously for 18-24 hours post coronary angioplasty[25]. Patients receiving heparin fared worse and developed significantly more IH than those receiving dextrose. A similar outcome resulted in the termination of a randomized trial comparing long term heparin administration subcutaneously with a control group after coronary angioplasty[26]. These results inevitably lead to disbelief of heparin as an agent suitable of controlling IH. However, Edelman and Karnovsky criticized the conduction of these trials, as doses, duration and type of administration did not correspond to earlier laboratory reports. When the clinical circumstances of these studies were applied to a laboratory setting, increased IH was observed there as well[82].

In 1994 a paper comparing LMWH (dalteparin) versus aspirin and dipyramidole after femoropopliteal bypass grafting was published[27]. Dalteparin 2500

IU was administered subcutaneously once daily for three months. However, all patients including the aspirin + dipyramidole group received dalteparin the first week after surgery. As animal experiments have indicated, this period is decisive with regard to preventing IH, and thus the methodology and validity of the study must be questioned. It was concluded that dalteparin was better in maintaining patency in a subgroup of patients with critical limb ischemia (CLI).

Another LMWH, enoxaparin, was investigated in the Enoxaparin Restenosis (ERA) Trial[29]. A randomized, controlled multicenter study (nine clinical centers) including a total of 458 patients, that sought to determine if 40 mg enoxaparin subcutaneously, once daily for one month after coronary angioplasty would reduce restenosis over six months follow-up. The conclusion was that it did not reduce restenosis. Neither did the EMPAR study investigating the effect of fish oils and enoxaparin 30 mg twice daily compared to a control group after coronary angioplasty[83]. The trial included 814 patients of whom 653 were randomized to enoxaparin or control. Following these two negative clinical studies, contrasting the results of animal experiments, Cairns suggested "*that species differences may preclude a beneficial effect of LMWH in humans*".

In the FACT (Fraxiparine Angioplastie Coronaire Transluminale) study, the LMWH nadroparin (Fraxiparine) was investigated in a multicentre double-blind randomized trial designed to compare the effects of treatment with nadroparin or aspirin on the occurrence of restenosis after coronary balloon angioplasty (available as an abstract only, 751-4, JACC, Febr 1995). 354 patients were randomized. Nadroparin 0.6 ml was administered subcutaneously for three months. Control angiography was performed in 91 % and revealed restenosis (>50 %) in 41 % of patients in the nadroparin group versus 38 % in the aspirin group, not statistically different.

The Subcutaneous Heparin and Angioplasty Restenosis Prevention (SHARP) trial was designed to determine whether unfractioned heparin, 12500 IU twice daily for four months would reduce clinical events and angiographic restenosis following coronary angioplasty or not[84]. 339 patients were included in this randomized multicenter trial. Patients either received heparin or not. No favourable effect was

seen in the heparin group.

In 2006 Koppensteiner et al. performed a clinical study including 275 patients undergoing transluminal angioplasty of femoropopliteal arteries[28]. One group received 2500 IU subcutaneously of the LMWH dalteparin plus aspirin for three months, the control group receiving only aspirin. No reduction of restenosis or reocclusion was observed. However, in the subgroup of patients treated for CLI, restenosis was observed significantly less frequent. This corresponds with the findings of Edmondson et al.[27].

Comment

These clinical studies demonstrated dismal effects of heparins in a clinical setting. Ellis and Lehmann reported a worse outcome in the heparin group, while the others demonstrated no effect or effect only in a subgroup (CLI). It is of notice that all but two studies were performed on patients undergoing coronary angioplasty. Both studies of peripheral vascular surgery found dalteparin to be effective in the subpopulation with CLI. Why dalteparin has proven effective in patients with CLI has not been settled. This may be due to enhancement of distal run-off by the added anticoagulation, vascular dilatation or the anti-inflammatory effects of heparins[28].

It is difficult to draw conclusions from such a heterogenic collection of studies, but nevertheless these have led to the conclusion that heparin does not provide any beneficial effects with regard to IH in a clinical setting. Compared to the laboratory studies, doses have generally been low and combined with intermittent administration. Duration of treatment had a time span of under 24 h to four months. Even if Edelman and Karnovsky criticized the conduction of clinical trials as early as 1994, later trials did not adjust doses, duration and type of administration to correspond more closely to previous laboratory reports.

1.7.3 Heparin Coated Grafts

The idea to improve the patency of prosthetic bypasses by a luminal coating with heparin was presented in the early nineties. The first models had heparin molecules bound ionically to the innermost layer of the prosthetic wall[19,21,22,85,86]. The ionic bond

proved unstable and the heparin coating was washed out within hours of implantation. A Swedish firm (Carmeda®, Upplands Väsby, Sweden) developed a more stable bond between heparin and the polytetrafluoroethylene (PTFE) surface. The Carmeda BioActive Surface (CBAS®) technology is based on single end-point covalent bonding of the heparin molecule to the inner surface of the prosthesis. The heparin molecule is immobilized, but still allows for the binding of antithrombin III (ATIII), thus retaining its anticoagulant properties to the prosthetic surface as long as neointimal coverage has not taken place. Clinical studies of the CBAS®-PTFE graft (Propaten®, W. L. Gore & Associates, Inc., Flagstaff, AZ, USA) have later presented results superior to uncoated ePTFE and similar to saphenous vein, although this is with regard to patency and not IH *per se*[87,88]. These publications were not available when the first part of this study (Paper I) was performed[17].

2. Aims of the Thesis

The aim of the thesis was to investigate certain processes of intimal hyperplasia. The specific aims were:

- To investigate if heparin-coated vascular grafts can improve patency and recruit less intimal hyperplasia compared to uncoated grafts grafts after six months (**Paper I**).

- To investigate if co-culturing endothelial cells with vascular smooth muscle cells produce results different from those of vascular smooth muscle cell monocultures with regard to proliferation (**Paper II**).

- To investigate the dose-response relationships of different heparins on proliferation, migration and signalling of patient-derived vascular cells (**Papers II & III**).

- To investigate if unfractioned heparin produces results different from those of low-molecular weight heparins on proliferation, migration and signalling of patient-derived vascular cells (**Paper III**).

- To determine the annual incidence of readmittance due to acute obstruction of aortic valve prostheses caused by annular intimal hyperplasia (pannus) (**Paper IV**).

- To investigate risk factors associated with development of annular intimal hyperplasia (pannus) (**Paper IV**).

3. Material and Methods

3.1 Paper I

3.1.1 Animals

Sheep were chosen for this study as an experimental protocol for vascular prostheses in sheep already existed and was approved by the National Committee for Experimental Studies on Animals in Norway. This model uses of the carotid arteries in sheep for in- and explantation of vascular grafts in order to evaluate the graft-properties. A similar technique was reported from a study performed in Vienna, Austria, in which different anastomotic techniques were studied[89]. Sheep carotid arteries have also previously been used for vascular graft implantation[90]. A pilot study on two animals was performed, concluding that the femoral and popliteal arteries did not have sufficient diameter to receive an artificial vascular prosthesis. On this basis the carotid arteries were chosen.

Fourteen female genetically secured Norwegian domestic sheep derived from Dalsau, Texel and Spelsau were used. Mean weight was 82 kg (range 68 – 97 kg) and mean age was five years. The sheep were bred on a farm nearby Haukeland University Hospital. The farm had teaching facilities and a veterinarian service that ensured that the sheep were kept healthy. The animals selected were female sheep with reduced breeding-potential or bad behaving as mothers. If not enrolled in this study, they would have been put down immediately. The surgical procedures were performed in the animal laboratory at Haukeland University Hospital. In between the surgical interventions, the animals were living under natural conditions at the farm.

3.1.2 Anaesthesia

After seven days of acclimatization in the veterinary facility at Haukeland University Hospital, the sheep were ready for surgery. Anaesthesia was induced through an intramuscular injection of medetomedine (Domitor®, Pfizer Animal Health, New York, NY, USA), ketamin (Ketalar®, Pfizer) and atropine. The anaesthesia was maintained throughout surgery with Isofluorane inhalation after endothracheal

intubation. Graft infection prophylaxis was administered once daily through an intramuscular injection of a combination of prokainpenicillin and dihydrostreptommycin 1 ml/10 kg (Streptocillin vet®, Boehringer Ingelheim GmbH, Ingelheim, Germany). The injections started preoperatively and continued for two days after surgery. Heparin 5000 International Units were administered intravenously before clamping the arteries. Postoperatively, no further antithrombotic medication was given.

3.1.3 Surgical Procedures

The common carotid artery was bypassed bilaterally in an end to side fashion with 6 cm long PTFE grafts, each with an internal diameter of 6 mm diameter and 25 µm porosity (W.L. Gore & Associates). The anastomoses were standardised by cutting the end of the graft in a 45° angle. The anastomoses were sewn end-to-side with continuous polypropylene 5-0 sutures (Prolene®; B Braun, Melsungen, Germany). After completion of the bypass, the common carotid artery between the proximal and the distal anastomosis was suture-ligated using 3-0 polypropylene (Prolene®; B Braun). Each animal received one PTFE-graft and one CBAS® -PTFE graft right/left in a random fashion. The order of graft implantation was randomized. Transit time flow measurements and intraarterial pressure measurements were performed as intraoperative quality control. All anastomoses were performed by two specialists in vascular surgery.

During the six-month period between implantation and explantation, the animals lived under natural conditions. Harvesting of grafts with adjacent artery was performed on living animals under general anaesthesia. The animals were subsequently put down with an intra-cardiac injection of potassium chloride. The cadavers were collected and further processed by Norsk Protein (Ingeberg, Norway).

3.1.4 Graft Specimens

Following explantation the grafts were flushed with isotonic saline water. Grafts with complete loss of lumen at explantation were regarded as occluded. The ventral part of the graft and the adjacent artery was longitudinally incised for exposure of the

anastomosis and the graft interior. One centimetre of the midgraft portion was excised for analysis of heparin bioactivity. The remaining parts of the explants were fixated in formalin 4 % for three days. The specimens were then dehydrated in alcohol (70 %) for one week and subsequently embedded in paraffin. Microscopic sections of the anastomoses were cut (Fig. 5: perpendicular to the graft axis with a thickness of 7 µm (Shandon® rotation microtome; Shandon Scientific ltd., Cheshire, UK)) and studied using a Zeiss Axio® ImagerA1/M1 light microscope (Carl Zeiss Imaging Solutions GmbH, Hallbergmoos, Germany). Digital photographs from the light microscope were taken using the software AxioVision 4.5 (Carl Zeiss Microimaging GmbH, Jena, Germany).

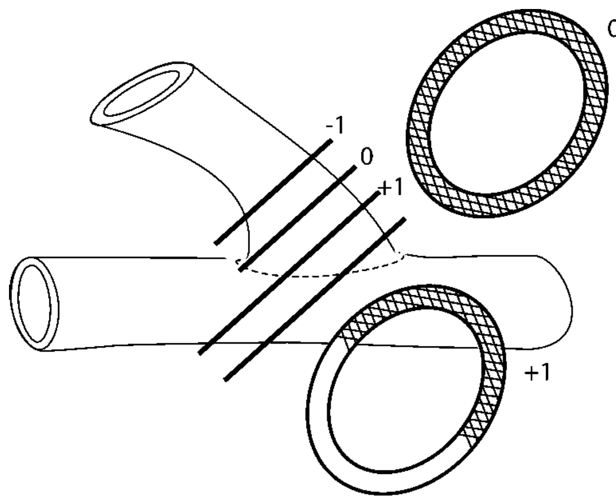

Figure 5. Preparation of the anastomotic area for micrographic analysis.
The "0" cut traverses the graft at 90° through the heel of the anastomosis. For analysis of IH in the entire anastomosis, additional cuts as indicated were made. Illustration courtesy of Prof. Erney Mattsson.

3.1.5 Heparin Bioactivity

Heparin activity of the explanted grafts (both CBAS® -PTFE and standard PTFE) was measured by exposing the specimen to an antithrombin (AT) solution. The reduction of AT content in the solution was proportional to the heparin activity of the graft, i.e. the amount of AT bonded to the immobilized heparin. The remaining AT concentration in the solution was then measured. Heparin activity was expressed as picomol/cm^2. The measurements were performed without knowledge of graft type.

3.1.6 Histomorphometrical Analyses

Staining prior to histomorphometrical analyses was performed with Haematoxylin-Eosin (H-E). In retrospect von Giessen staining would have been preferable as membrana elastica interna is better visualized, thereby making demarcation of the IH easier and more accurate. This was unknown at the time of analysis. Even so, as the micrographs were evaluated quantitatively using the digital software Biopix® (Biopix, Gothenburg, Sweden), accuracy seemed addressed (Fig. 6). Mean intimal thickness was analysed in all open grafts. Calculation was performed by dividing the total IH area with graft length and expressed as mean values. Mean intimal thickness at the anastomoses of open PTFE versus open CBAS®-PTFE grafts were compared.

3.1.7 Statistics

Categorical data for groups were analysed with Fisher's exact test. The Mann-Whitney U-test was applied for comparison of IH between control PTFE and CBAS®-PTFE grafts. Paired t-test was used for comparison of heparin bioactivity measurements. SPSS 13.0 for Windows (IBM Company Headquarters, Chicago, IL, USA) was used for statistical analyses. Probability values of ≤ 0.05 were considered significant.

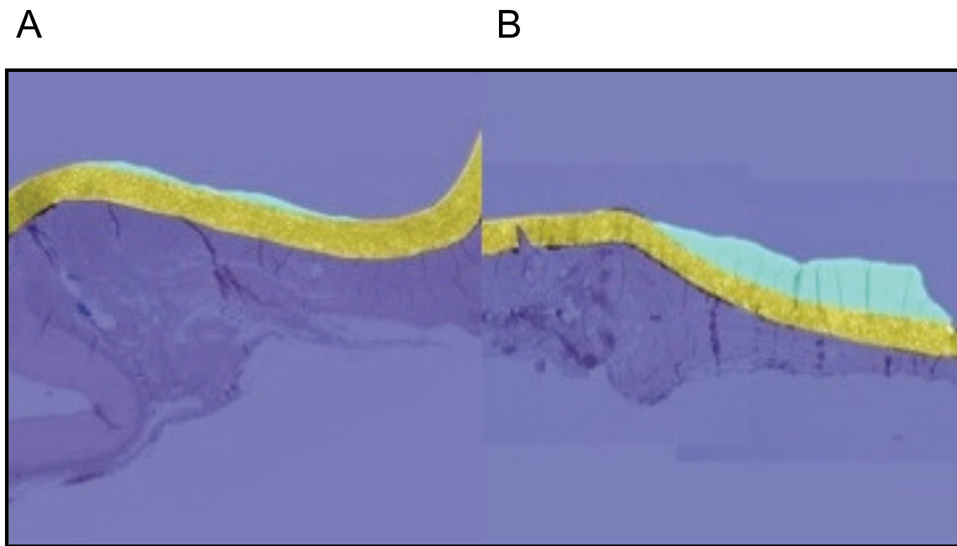

Figure 6. Micrographs of intimal hyperplasia evaluated quantitatively using Biopix® digital software.

Intimal hyperplasia formation (light blue) on PTFE grafts (yellow) ranged from scarce (A) to extensive (B).

3.2 Paper II & III

3.2.1 Vascular Biobank

Approval to establish a human tissue biobank and to perform the experimental studies of Paper II & III was granted by the Regional Independent Scientific Ethical Committee and the Norwegian Directorate of Health. As no method for establishing cell-cultures from vascular biopsies existed at the laboratory, a new technique for isolating and culturing ECs and VSMCs was developed.

3.2.2 Patient-Derived Vascular Cell Isolation

Full-thickness tissue biopsies from the aortic wall were taken from six consenting patients during vascular surgery (Table I). Isolation of cells was based upon a method described by Bryan and D'Amore[91]. Tissue samples were surgically divided into tunica media and tunica intima, and treated separately. The tissue was finely cut and mixed with phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) to separate cells from surrounding tissue. The cell suspension was centrifuged at 515 x g for five minutes and the pellet resuspended in 0.2 % collagenase bovine serum albumin (BSA; Sigma-Aldrich). The cells were incubated for 30 minutes in 5 % CO_2 at 37˚C before 0.25 % Trypsin Ethylene-diamine-tetraacetic acid (EDTA; Sigma-Aldrich) was added. The cells were then incubated for another 15 minutes under the same conditions. The cell suspension was centrifuged at 515 x g for 15 minutes before the pellet was resuspended in 300 µL cell specific growth medium, transferred to a cell culture plate and placed in an incubator. VSMCs from biopsied tunica media were cultured in hFGF-supplemented smooth muscle growth medium-2 (SmGM-2; Lonza, Cologne, Germany) and ECs from the tunica intima in vascular endothelial growth factor (VEGF)-supplemented endothelial cell growth medium-2 (EGM-2; Lonza). After 2-6 days of incubation the proliferating monolayers of cells were examined, sorted and validated.

Table I. An overview of patient characteristics and comorbidity

Patient	Gender	Age	Aorta	HT	DM	HChol	Smoke	Morbidity
1	Male	22	Thx	Yes	No	No	No	Coarctation
2	Female	58	Thx	Yes	No	No	No	TAA, AVR
3	Male	64	Abd	No	No	No	Earlier	COPD, AAA
4	Female	83	Abd	No	No	No	No	AAA
5	Male	41	Thx	No	No	No	No	TAA
6	Female	62	Thx	Yes	No	No	Earlier	TAA, AVR

Abbreviations: Thx=thoracalis; Abd=abdominalis; HT=hypertension; DM=diabetes mellitus; HChol=hypercholesterolemia; TAA=thoracic aortic aneurysm; AAA=abdominal aortic aneurysm; AVR=aortic valve replacement; COPD=chronic obstructive pulmonary disease.

3.2.3 Sorting and Validation of Patient-Derived Vascular Cells

The cells were sorted with CD31 Dynabeads, Endothelial Cell (Invitrogen 111.55D; Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The cells were washed with PBS (Sigma-Aldrich) and trypsinized with 0.25 % Trypsin-EDTA, before 5 % fetal bovine serum (FBS; PAA Laboartories, Pasching, Austria) in PBS (Sigma-Aldrich) was added. The cells were then centrifuged at 201 x g for 5 minutes before being resuspended in 0.1 % BSA (Sigma-Aldrich) in PBS (Sigma-Aldrich) to yield approximately two million cells in each tube. 25 µL of Dynabeads per mL of cell suspension were added before being placed on a carousel and incubated at 4 ˚C for 20 minutes. Thereafter 0.1 % BSA (Sigma-Aldrich) in FBS (PAA Laboartories) was added and the tubes placed on ice in the Magnetic Particle Concentrator (Life Technologies) for two minutes before the supernatant containing $CD31^-$ VSMCs was collected. The $CD31^+$ cells bound to the beads contained ECs and contaminating fibroblasts. To separate the ECs from the fibroblasts, *Ulex europaeus* agglutinin I (UEA-I)-lectin (Sigma-Aldrich) conjugated

to tetramethyl-rhodamine isothiocyanate (TRITC, L4889; Sigma-Aldrich) was added to the cell culture, thereby staining the ECs. The ECs were separated from the lectin-negative fibroblasts on an Aria fluorescence-activated cell sorter (FACS; BD Biosciences, San Jose, CA, USA) and verified by expressing CD31, intercellular adhesion molecule 2 (ICAM-2, ab35045; Abcam, Cambridge, UK) and bound UEA-I lectin. The VSMCs were verified by anti-SMC actin M0851 Western Blot (Dako, Glostrup, Denmark).

3.2.4 EC-VSMC Co-Culture Method

An EC-VSMC co-culture method was applied to study the effects of heparin in a model more representable of *in vivo* conditions. It generates *in vitro* organotypic capillary-like structures comprising basement membrane and pericyte enveloped three dimensional endothelial tubular structures[92]. This approach has previously been used to study several facets of angiogenesis, vascular homeostasis and antivascular compounds[93-100]. Patient endothelial cells self-assemble into capillary-like structures embedded in a locally deposited collagen and laminin rich matrix in the presence of VSMCs (Fig. 7). VSMCs reciprocally express VEGF that drives EC morphogenesis[91]. As ECs stop dividing, only the VSMCs will proliferate and contribute to cell number increases[92].

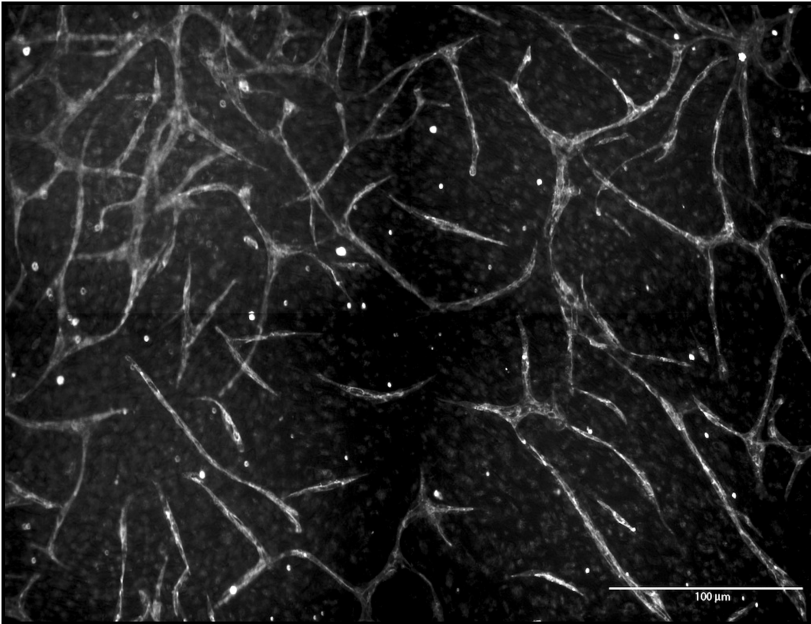

Figure 7. EC-VSMC co-culture.

Micrograph of the capillary-like network at 3 days formed by ECs co-cultured with VSMCs from the same patient. Scale bar, 100 microns.

3.2.5 Cell Assays

3.2.5.1 Proliferation

The BD Pathway system 855 (BD Biosciences, San Jose, CA, USA) is a highly accurate imaging device for quantification of cell nuclei by direct enumeration.

The cells were serum starved over night in smooth muscle basal medium-2 (SmBM-2, Lonza) + 0.2 % BSA (w/v; Sigma-Aldrich) before cultures of VSMCs and EC-VSMCs were set up in 96-well plates. The total number of cells seeded out was maintained at 2.5 x 10^3 for both mono- and co-cultures. VSMCs were cultured in hFGF-supplemented SmGM-2 (Lonza). The co-cultures were seeded out in a 50:50 ratio of ECs and VSMCs and grown in EGM-2 (Lonza). All experiments were run in triplicate. All cell cultures were incubated in 5 % CO_2 at 37 ˚C for two hours before heparin (100 IU/mL; LEO Pharma, Ballerup, Denmark), enoxaparin (Klexane®, Sanofi-Aventis) and dalteparin (Fragmin®, Pfizer) were added in the concentrations

of 0, 5, 10, 25, 50 and 100 μg/mL. Incubation was further continued for 96 hours.

Cell numbers were determined at assay endpoints by automated image acquisition and image analysis employing the BD Pathway system 855 (BD Biosciences). Prior to the analysis the system was set up and calibrated by a company technician. Cell cultures were stained with Hoechst solution 10 ng/mL (B2261; Sigma-Aldrich) for 15 minutes. Images were acquired with 20x objective as 2x2 montages and the total number of nuclei (Hoechst) was quantified using the Montage cell-count algorithm embedded in Attovision v1.6.2 (BD Biosciences). The measurements resulted in a dose-response curve for each cell culture.

3.2.5.2 Migration

The xCELLigence™ system (Roche Applied Science, Penzberg, Germany) with CIM 16 migration analysis plates was used for automated real-time migration monitoring. The system was set up and calibrated according to the producer's specifications.

160 μL of complete SmGM-2 (Lonza) was added to the lower chamber wells of the CIM-plate. The top chamber, containing the membrane and electrodes, was attached before 50 μL SmBM-2 (Lonza) with 0.2 % (w/v) BSA (Sigma-Aldrich) was added to the top chamber wells. The plate was then incubated (5 % CO_2 at 37 °C) for one hour to allow the CIM-plates' membrane surface to reach equilibrium with the media. The spontaneous background activity was measured. 10 000 cells that had been serum starved over night in 100 μL SmBM-2 (Lonza) and 0.2 % BSA (Sigma-Aldrich) were added to the top chamber wells together with 0, 5, 10, 25, 50 and 100 μg/mL of heparin, enoxaparin or dalteparin. Each concentration was run in quadruplicate. The prepared CIM-plates were left in room temperature for 30 minutes for the cells to settle on the upper side of the membrane. The plates were then loaded into the xCELLigence™ system and the scan period started. Scanning was set to incur every 10 minutes for the first four hours and every 15 minutes the next 20 hours. Plateau phase was reached after 100 minutes. The measurements were plotted as the relative change in measured electrical impedance of the chamber-electrodes as cell index.

3.2.5.3 Flow Cytometry

Cold trypsin phosphorylation specific flow cytometry was used to study the response of the MAPK-ERK pathway to varying concentrations of heparin, enoxaparin and dalteparin[101-103].

Patient-derived VSMCs were grown in 6-well culture dishes (Fig. 8). The cells were serum starved overnight before stimulated with hFGF (R&D systems Inc., Minneapolis, MN, USA) at the concentration of 100 ng/mL for 15 minutes. The cells were put on ice immediately after stimulation to quench cell signalling. The cells were then washed twice with ice cold PBS before being trypsinized using 0.5 % of Trypsin-EDTA at 4 °C to avoid protease-dependent activation of cell signalling responses. 16 % paraformaldehyde (PFA) solution (Electron Microscopy Sciences, Hatfield, PA, USA) was added directly to the cell suspension to obtain a final concentration of 1.6 % PFA. The cells were incubated in the fixative for 20 minutes at room temperature before being washed with PBS (Sigma-Aldrich) and pelleted before permeabilized in 100 % cold methanol (Sigma-Aldrich).

To stain the cells for flow cytometry, the samples were first centrifuged at 448 x g for five minutes to remove the methanol and then washed with FACS buffer (1 % weight/volume BSA in PBS). Cells were then stained with p44/42 MAPK antibody 4695 (Cell Signaling, Danvers, MA, USA) at a dilution of 1:1000 for 30 minutes at room temperature. The cell suspension was then washed with FACS buffer before goat anti-rabbit IgG-Alexa Fluor 647 (A-21244, Invitrogen; Life Technologies) was added at a concentration of 1:2500 (0.8 μg/mL) as a secondary antibody. The cells were incubated for 1 hour in darkness at room temperature before being washed once more with FACS buffer. In order to verify phosphorylated MAPK-ERK signal specificity, cells were treated with the mitogen activated protein kinase kinase (MEK1) inhibitor PD98059 (Cell Signaling) at a concentration of 30 μM for three hours. Samples were run on BD Accuri C6 (BD Biosciences) to determine the phosphorylation levels of MAPK-ERK. Analyses were performed using FlowJo (TreeStar Inc., Ashland, OR, USA).

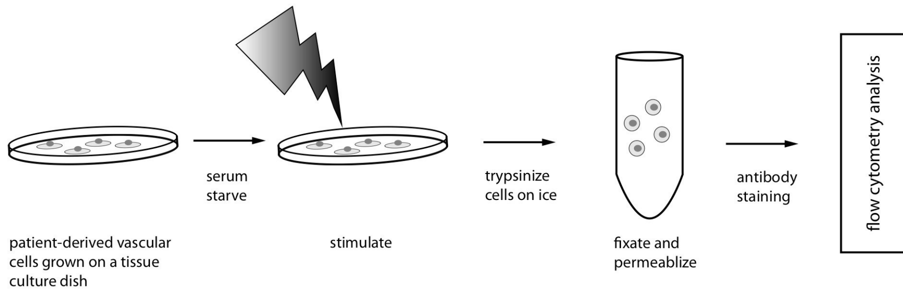

Figure 8. Schematic overview of the flow cytometry approach.
Adherent patient-derived VSMCs were harvested by a cold trypsinization approach to minimize protease activated cell signalling. Recovered cells were fixed and permeabilized, and stained with anti-pERK antibodies followed by fluorescently conjugated (AlexaFluor 647) secondary antibodies. Flow cytometry was conducted to quantify the anti-pERK dependent fluorescence levels in individual patient VSMCs. Illustration reproduced with permission from Iren Abrahamsen.

3.2.6 Statistical Analysis

To detect differences of proliferation, migration and signalling as a function of concentration, linear regression analysis of fitted trend lines was used. The trend lines were fitted using the least-squares method and tested for linearity. IC50 was calculated using the fitted trend line formula.

For the proliferative assay, each patient culture sample was analysed in triplicate during two separate experiments for each concentration. Data were normalized to untreated control values by calculating the coefficient needed for the automated image-based cell enumeration at 0 µg/mL to equal 100.

For the migration assay, each sample was analysed in quadruplicate. The cell indices at 100 minutes were normalized to untreated control by calculating the coefficient needed to achieve a cell index of 0.35 at 0 µg/mL.

For the flow cytometry, three series were analysed. The MAPK-ERK phosphorylation levels were normalized to untreated control.

Probability values of <0.05 were considered significant throughout the studies. All statistical analyses were done using Excel® for Mac 2011 (Microsoft Corporation, Redmond, WA, USA) and STATA for Mac (StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX, USA: StataCorp LP).

3.3 Paper IV

3.3.1 Material

From 1982 to 2004, 1187 patients underwent aortic valve replacement with the Medtronic-Hall monoleaflet valve prosthesis (Medtronic, Minneapolis, MN), which was the preferred heart valve prosthesis at the Section of Cardiothoracic Surgery, Department of Heart Disease, Haukeland University Hospital, Bergen, Norway at that time. All patients were registered in the department database. Upon readmission, signs and symptoms, diagnostic procedures, perioperative findings, and in-hospital complications were registered. Retrospective patient information was taken from medical records. Pannus overgrowth was ascertained either at reoperation or autopsy. The endpoints were reoperation and death.

3.3.2 Statistics

T-test was used to compare means of continuous variables. Relationships between gender, valve size and pannus were assessed by Pearson's χ^2 test and multivariate regression analysis. One-way analysis of variance (ANOVA) was used to compare multiple means. Increased risk was quantified by using the odds ratio (OR). Probability values of <0.05 were considered statistically significant for all tests. The analyses were performed using STATA for Mac (StataCorp. 2011. *Stata Statistical Software: Release 12*).

4. Summary of Results

4.1 Paper I

The aim of this study was to investigate if CBAS®-PTFE grafts could improve patency and recruit less IH compared to control PTFE grafts at a longer term (six months) in a sheep model.

Twenty-eight common carotid arteries in 14 sheep were bypassed with a luminal heparin-coated PTFE graft on one side and a control uncoated PTFE contralaterally. The grafts were explanted after six months. The thickness of IH in open grafts was measured with histomorphometrical methods. In this first large animal carotid bypass model comparing conventional ePTFE with CBAS®-PTFE, the latter performed better with respect to patency at six months and reduction in IH growth. Two of 14 heparinized PTFE grafts and nine of 14 grafts in the control PTFE-group were occluded at explantation (P=0.006). Six months patency rates for CBAS®-PTFE grafts and for standard PTFE grafts were 86 % and 36 %, respectively. Mean graft anastomotic IH thickness in open grafts were 0.074 mm for CBAS®-PTFE and 0.259 mm for PTFE-grafts (P=0.006).

In conclusion, PTFE grafts with luminal coating containing immobilized heparin had significantly better patency and recruited less IH than standard PTFE grafts at six months.

4.2 Paper II

This study was designed to investigate the influence of heparin on the processes of IH of patient-derived vascular cell mono- and co-cultures. VSMCs and ECs were isolated from vessel wall biopsies from six patients. The cell cultures were treated with heparin and evaluated for effects on proliferation, migration and MAPK-ERK signal transduction using image-based cell enumeration, real-time migration monitoring and flow cytometry.

Heparin inhibited proliferation and migration of patient-derived VSMC cultures, but only at concentrations exceeding clinical doses. The proliferative response of VSMCs to heparin was not affected by the presence of ECs. Heparin

stimulated MAPK-ERK phosphorylation of VSMCs at lower concentrations in the presence of hFGF (Fig. 9). MAPK-ERK phosphorylation of unstimulated cultures was inhibited dose-dependently. Thus, heparin seems to influence patient-derived vascular cells through both a stimulatory hFGF-dependent and an inhibitory hFGF-independent pathway. These findings may explain the divergence of results between previous *in vitro* and clinical studies and provide a basis for new therapeutic strategies.

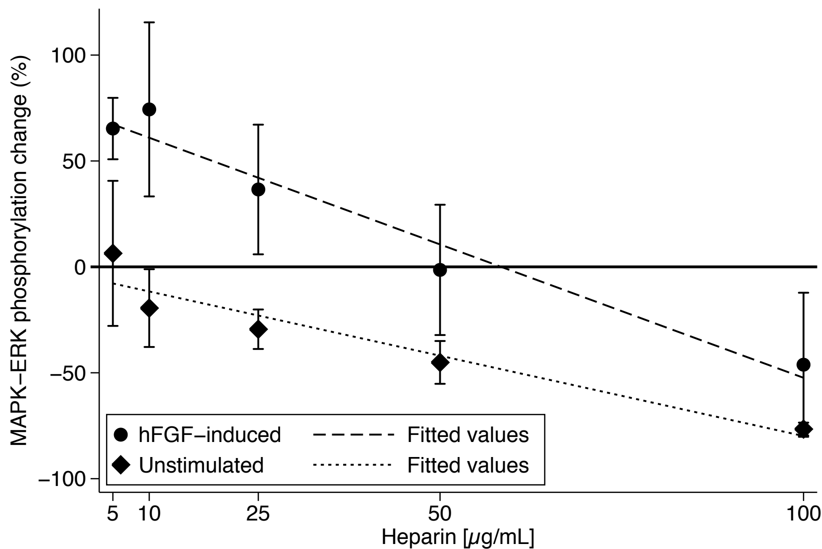

Figure 9. Effects of heparin on unstimulated and hFGF-induced MAPK-ERK phosphorylation of patient-derived VSMCs

MAPK-ERK phosphorylation of patient-derived VSMCs treated by heparin as assessed by flow cytometry. Heparin concentrations (µg/mL) are along the abscissa and MAPK-ERK phosphorylation change (%) relative to untreated control along the ordinate. There was an increase of MAPK-ERK phosphorylation at lower concentrations when induced by hFGF (100 ng/mL). MAPK-ERK phosphorylation of unstimulated cultures was inhibited dose-dependently. This suggests that the cellular response to heparin is based on the activation-ratio between a stimulatory hFGF-dependent pathway and an inhibitory hFGF-independent pathway.

4.3 Paper III

This study was designed to investigate the effects of enoxaparin and dalteparin compared to unfractioned heparin on proliferation, migration and MAPK-ERK signal transduction of patient-derived VSMCs. VSMCs were treated with the two LMWHs in a range of concentrations and evaluated using image based cell enumeration, real time migration monitoring and flow cytometry. Series treated with unfractioned heparin were included as positive controls and untreated series as negative controls.

Neither enoxaparin nor dalteparin influenced proliferation and MAPK-ERK phosphorylation. Migration was reduced slightly by both LMWHs. Unfractioned heparin exhibited dose-dependent effects different from those of the LMWHs in all analyses. Thus, this study demonstrated a difference in proliferative and migratory effects between the two LMWHs and unfractioned heparin in patient-derived VSMCs. The effects corresponded to the MAPK-ERK phosphorylation, suggesting different mechanisms of action. These results may explain why clinical trials using LMWHs to prevent IH have failed to observe a reduced incidence of restenosis and do not support prolonged therapeutic use to prevent it.

4.3 Paper IV

The aim of this study was to analyse the incidence, treatment and results of acute prosthetic valve obstruction due to pannus overgrowth in a large series of patients with a Medtronic-Hall tilting disc aortic prosthesis (Medtronic). Pannus is recognized as IH originating from the periannular area. This was a retrospective study of patients treated at Haukeland University Hospital during the last 30 years. Special attention was given potential predisposing factors of pannus development.

Twentyseven (2.3 %) of the 1187 operated patients had been readmitted due to acute obstruction caused by pannus, resulting in an annual incidence of 0.7 per 1000. Median time from primary operation to valve dysfunction was 11.1 years (range: 1.2-26.8 years; Fig. 10).

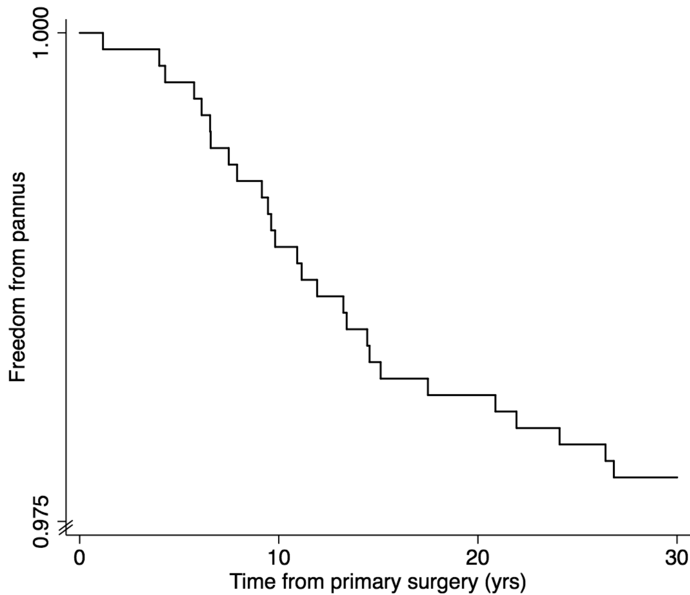

Figure 10. Incidence of pannus obstruction by time from primary surgery. *Kaplan-Meyer plot of freedom from valve obstruction due to pannus after implantation of a Medtronic-Hall aortic valve prosthesis.*

Pannus obstruction occurred with all implanted valve sizes, except 31 mm (Fig. 11). Median size of obstructed valves was 23 mm (size range: 20-29 mm). Valve size was not an independent risk factor for pannus obstruction in our sample. Females had higher risk for pannus overgrowth than males. Patients developing pannus with acute valve dysfunction were younger at the time of the primary valve implantation than the rest of the Medtronic-Hall aortic valve cohort. Of the 20 patients that were reoperated, two died. Seven patients died before reoperation.

In conclusion, females and younger patients are at higher risk for pannus development. When acute dysfunction by pannus is suspected in a mechanical aortic valve, an immediate echocardiogram and an emergency aortic valve replacement should be carried out due to the potential of fatal outcome.

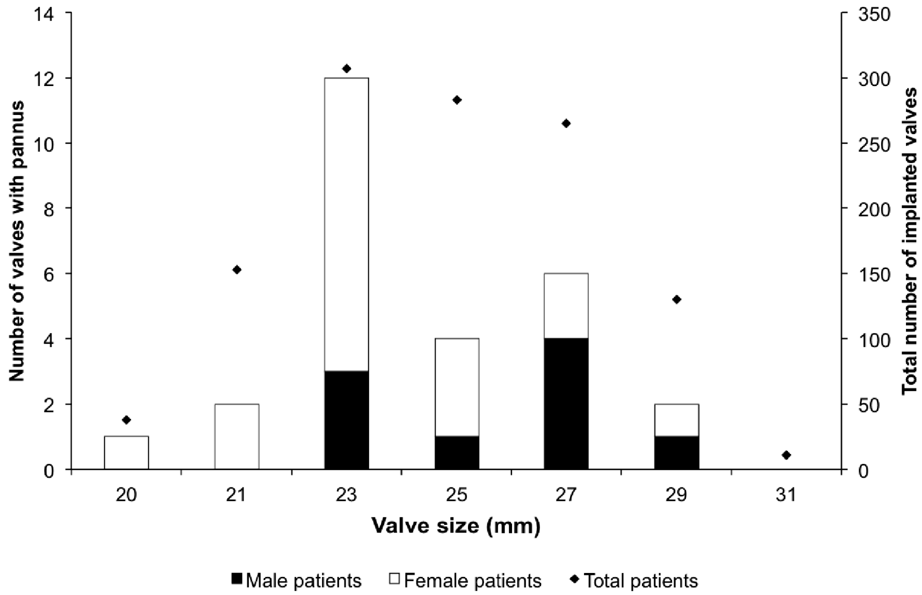

Figure 11. Distribution of valve sizes.

Bars show the distribution of Medtronic-Hall aortic valve prostheses with pannus obstruction, stratified by size and gender. Diamonds represent the total number of implanted Medtronic-Hall aortic valves by size.

5. Discussion

5.1 Paper I

5.1.1 General Discussion

At the time of preparing this study, only a limited number of clinical papers on the heparin-coated CBAS®-PTFE vascular graft were published[104-106]. Patency and limb-salvage were end points. The initial results were promising and comparable with the golden standard: autologous vein. Reduction of IH was thought to be one of the contributing factors as seen in animal studies[15,16,107]. These studies, investigating the efficacy of CBAS®-PTFE grafts, had follow-up ranging from four weeks to three months postoperatively. IH develops and remodels during a longer timespan. The aims of this study were therefore to investigate if CBAS®-PTFE grafts could improve patency and recruit less IH compared to control ePTFE grafts at a longer term (six months) in a sheep model.

By investigating patency in addition to IH-formation *per se,* other aspects were also addressed. Inhibition of platelet activation and aggregation, anticoagulation, and inhibition of the inflammatory response were all thought to contribute to the improved patency by heparin. Heparin reduces platelet aggregation, thus reducing the amount of PDGF in the graft surroundings[108]. As PDGF is a powerful chemoattractant for VSMCs, less activation of these cells results. The anticoagulative mechanism of heparin is mainly in its binding to ATIII, which inhibits activation of platelets. Heparin has long been known to inhibit inflammation and has a potent antiproliferative effect on VSMCs[8].

5.1.2 Intimal Hyperplasia - IH

It may be debatable whether or not the statistical difference in IH thickness between the two graft types was of clinical significance. A 0.259 mm thickening in a 6 mm graft at six months yields only a modest reduction in diameter (8.83 %). However, any reduction in the formation of IH is clinically important, due to the fact that formation of IH may continue beyond six months and up to 18 to 24 months. Sustained heparin activity reduces luminal thrombus accumulation, thus decreasing

the scaffold available for anastomotic remodelling events. This may partly explain the reduced IH formation in the CBAS®-PTFE grafts investigated in this study.

5.1.3 Heparin Bioactivity

One aim was to assess heparin bioactivity and IH growth beyond the period of neointimalization of the graft. However, measurements of heparin activity of the explanted grafts were affected by the presence of neointima on the luminal surface of the graft. Attempts to remove the neointimal layer in order to perform these measurements were unsuccessful, because removal of neointima led to destruction of the heparin coating. The fact that all of the grafts in the series were completely covered with neointima suggests that the effect of the heparin coating is most important during the first weeks postoperatively.

5.1.4 Antiplatelet Medication

Use of an antiplatelet agent reduces the frequency of early prosthetic graft occlusions and improves patency[109]. The results of this study might have been different if the animals had been treated with an antiplatelet agent. However, administration of antiplatelet agents to animals living under natural conditions proved impossible.

5.1.5 Summary and Limitations

Overall, the results of this study were convincing, comparable to other animal studies and provided further support to the hypothesis that the luminal bound heparin reduced IH. However, previous studies of heparin signalling in animal cells suggested that heparin binds to high-affinity surface-receptors and undergoes endocytosis[67,68]. The internalized heparin concentrates in the perinuclear region. Since CBAS®-PTFE has covalently bound heparin on the luminal surface, internalization should be impossible. On the contrary, Majack et al. demonstrated that heparin causes the release of thrombospondin, suggesting that heparin affects VSMC proliferation and migration via an extracellular mechanism without being bound and internalized. This suggested mechanism could explain our findings, but a decade after these studies, Letourneur et al. published two papers, supporting Castellots findings that heparin is

internalized[78,79]. The covalently bound heparin of the CBAS®-PTFE graft would in theory be able to form surface-contact with the vascular cells, but internalization, intracellular metabolism and degradation is difficult to imagine.

The main limitation of Paper I was due to its animal nature, since it had not been validated if the results were translatable to human biology. To investigate these issues, Paper II was designed.

5.2 Paper II

5.2.1 General Discussion

As no detailed data on the effects of heparin in human vascular cells could be found, evaluation and interpretation of the contradictory results between previous human and animal experiments was impossible, as discussed in Section 1.7. The discrepancy of results may be due to several factors, but diverging experimental conditions are the most conspicuous. The *in vitro* studies were largely conducted using animal VSMC-lines and heparin concentrations exceeding the recommended therapeutic dosage. Even if heparin has largely been abandoned in the treatment of IH, further investigation of its known inhibitory effects can contribute to a deeper understanding of the pathophysiology and unveil new therapeutic principles. Thus, this study was designed to test if heparin inhibits proliferation, migration and MAPK-ERK signalling of patient-derived vascular cells at clinically relevant concentrations.

5.2.2 Proliferation

This study demonstrated that heparin inhibited proliferation of patient-derived mono- and co-cultures, but only at concentrations far exceeding acceptable therapeutic dosages. The antiproliferative effect of heparin has previously been demonstrated in studies on both animal and human cells, but high concentrations have generally been used. Chan et al. is the exception, including 10 μg/mL in their setup[59]. Their conclusion was that heparin had an antiproliferative effect, but only at 100 and 1000 μg/mL. At 10 μg/mL there was negative inhibition, i.e. stimulation of proliferation.

The putative influence of ECs on the heparin responsiveness of VSMCs has been attributed to nitrogen oxide (NO), growth factors and endogenous

glucosaminoglycans[11,37-39]. This was investigated by employing a vascular cell co-culture system that models reciprocal EC-VSMC interactions. VSMC proliferation was not significantly affected by the presence of ECs in this study. It can be argued that this result was as expected since cells were not damaged as they would be in case of surgery. Presence of damaged cells could have led to different microenvironmental conditions, producing a different outcome. Due to methodological constraints, it was not possible to introduce reproducible controlled damage corresponding to the actual surgical trauma. In previous experiments of heparin effects on human VSMCs by Chan and Refson, results were reproducible and significant despite the lack of injury[12,14,59]. On this basis it was concluded that a setup of cell cultures without introducing injury would provide reproducible and reliable data.

5.2.3 Migration

Only a few papers describe the migratory effects of heparins[10,69]. Considered one of the two main processes of IH, it is surprising to find such scarce amounts of experimental data on the subject. Majack et al. found heparin to inhibit migration of rat aortic muscle cells. IC50 was 1 µg/mL, while maximum inhibition was reached at 10 µg/mL. Clowes et al. observed a marked reduction of VSMC migration in heparin treated rats analysing [^{3}H]thymidine distribution in autoradiograms. With newer *in vitro* quantification methods availableit was possible to investigate the effects of heparins on migration in human cells. Migration of human VSMCs by heparin was reduced at 25 µg/mL and above. Correlating these results with those of Majack et al., the tendency is the same, but at different concentrations. A correlation to the study by Clowes et al. is difficult due to weight-calculated dosage and continuous administration in the rats. However, the conclusions are similar.

5.2.4 Signal Transduction

The proliferative and migratory results of these studies can be explained by the MAPK-ERK phosphorylation response to heparin. MAPK-ERK phosphorylation was increased at lower concentrations when stimulated by hFGF (100 ng/mL), while unstimulated cultures were inhibited dose-dependently (Fig. 9). Thus, heparin seems

to regulate MAPK-ERK phosphorylation of VSMCs through both a stimulatory hFGF-dependent pathway and an inhibitory hFGF-independent pathway. These observations suggest that the MAPK-ERK response of patient derived VSMCs to heparin relies on the activation-ratio between the two pathways. Hence, heparin treatment enhanced hFGF-induced MAPK-ERK phosphorylation of patient VSMCs at low concentrations and reduced at high. This may reflect the findings of clinical trials with no effect or worse outcome in patients treated with heparin[25,26,84]. This can also explain why previous experiments on animals and animal cells have found inhibitory effects on proliferation and migration only at higher concentrations.

5.2.5 Limitations

Due to the fact that this was an in vitro study trying to draw conclusions applicable *in vivo*, there are limitations. Contact with blood, pulsatile blood-flow, trans vessel-wall gradients and the local inflammatory responses were missing. This model was, however, a proposal for setting up an *in vitro* experiment that replicates the human *in vivo* conditions as far as possible, using state of the art methods.

The EGM-2 growth medium contained heparin. This may have preloaded and desensitized the co-cultures and thereby acted as a confounder. Though, it was considered insignificant since the concentration was very low.

Variation of results was observed, but as methods were as standardized and reproducible as possible to avoid confounding, it was concluded that this variation was caused mainly by biological variance.

The study was based on cells from six patients and all biopsies were from the aorta. Although the number of patients is low, this represents a selection of actual patients that underwent vascular surgery. Thus, this material should be more suited for analysis than laboratory grown animal cells, as generally used in previous studies. Biopsies from the femoral artery were also harvested, but did not yield ECs and were thereby excluded. Lack of EC growth was probably due to grave atherosclerosis in the operative region making the vessel dissection difficult. Testing cells from only one region makes generalization of the findings difficult. However, Refson et al. compared vein-, aortic- and peripheral arterial tissue with regard to the

antiproliferative effect of heparin and found the VSMCs to respond identically when coming from the same individual[14]. They also found interindividual variance, considered to reflect the individual's resistance to heparin. This study affirmed the interindividual variance.

5.3 Paper III

5.3.1 General Discussion

As demonstrated in Paper II, unfractioned heparin inhibits the two main processes of IH: proliferation and migration of VSMCs. Due to ease of administration, more easily predictable effects on metabolism and longer half-life, their low-molecular-weight derivates are preferred to unfractioned heparin in clinical practice and often regarded as synonymous substances. Previous studies using rat, minipig, rabbit and sheep models have demonstrated that LMWHs also inhibit IH processes[18-24]. In contrast to these results of animal experiments, LMWHs have repeatedly failed to reduce the level of restenosis in clinical trials[27-31]. It should be taken into consideration that the clinical trials were performed on varying study populations, using different types of LMWHs, doses, and administration protocols. Despite the lack of evident clinical benefit LMWHs are still used therapeutically for prolonged periods following vascular surgery and endovascular interventions with the intention of preventing IH. This study was designed to investigate the effects of LMWHs on proliferation, migration and MAPK-ERK signal transduction of patient derived VSMCs, and investigate if the effects differ from those of unfractioned heparin.

5.3.2 Proliferation

Neither enoxaparin nor dalteparin inhibited proliferation of patient-derived VSMCs in contrast to the positive heparin control. The exact mechanisms by which heparin influence proliferation and migration are still obscure. Interaction with an inhibitory heparin specific receptor mediated pathway or the stimulatory FGF pathway affects MAPK-ERK phosphorylation with subsequent proliferative and migratory effects[55,110]. The minimum length of heparin GAG-chains needed for FGFR dimerization is a hexasaccharide[111]. The minimum length needed for the binding and

activation of the heparin receptor is unknown. LMWHs inherently have shorter GAG-chains than unfractionated heparin and could have explained the lack of effect demonstrated by this study. However, the GAG length of the two LMWHs used was a mixture of 1-20 chains, in theory sufficient to influence the FGF pathway. The present results do not reflect such an effect, and may rather depend on the degree and distribution of GAG sulfatation reported to be of potentially greater importance than chain length *per se*[61].

5.3.3 Migration

Enoxaparin and dalteparin both had a slight inhibitory effect on migration, but less efficiently than the positive heparin control. The concentration needed to reach IC50 was much higher than what is consistent with systemic administration in clinical practice. Since no effect of the LMWHs on MAPK-ERK phosphorylation was observed, migration might be controlled through different pathways, e.g. p38 MAPK, as Boilly et al. suggested[112].

5.3.4 Signal Transduction

No dose-dependent effect of enoxaparin and dalteparin on MAPK-ERK phosphorylation was observed. This contrasts the results of the positive heparin control (Fig. 12), reflects the differences of proliferative and migratory effects and suggests different mechanisms of action.

5.3.5 Limitations

The same limitations as discussed in Section *5.2.5* apply to this study.

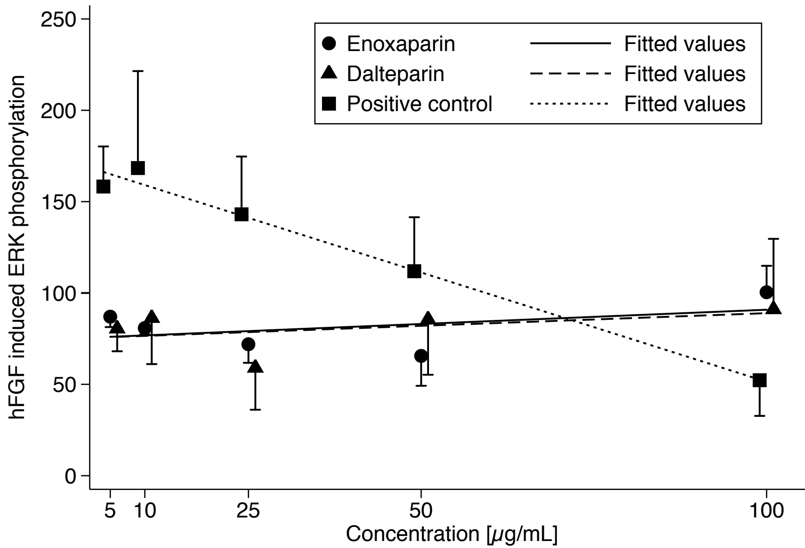

Figure 12. Influence of enoxaparin and dalteparin on hFGF induced MAPK-ERK-phosphorylation.

MAPK-ERK phosphorylation of hFGF induced patient-derived VSMCs treated with enoxaparin and dalteparin. Positive control by unfractioned heparin, untreated control=100. The LMWHs did not have any effect on MAPK-ERK phosphorylation in contrast to the positive heparin control, suggesting different mechanisms of action.

5.4 Paper IV

5.4.1 General Discussion

The most important observation of this study was that females had higher risk for pannus overgrowth than males. Svennevig et al. reported that female gender was a significant risk factor for death following aortic valve replacement, but possible reasons were not addressed[113]. Some authors speculate that the higher rate of death in female patients after aortic valve replacement may be due to implantation of smaller prosthetic valves, resulting in higher bloodstream velocity and increased turbulence[114-116]. However, in this study pannus obstruction occurred with all implanted valve sizes, except 31 mm valves (Fig. 11). Valve size was not an independent risk factor for pannus obstruction.

Patients developing pannus with acute valve dysfunction were younger at the time of the primary valve implantation than the rest of the Medtronic-Hall aortic valve cohort. Teshima et al. hypothesized that a chronic periannular inflammatory response to a foreign body triggers pannus development[6]. Younger patients are thought to produce stronger inflammatory reactions, stimulating a disproportionate overgrowth of pannus. They are also usually more physically active which may cause more mechanical and shear stress to the valvular region.

The 2.3 % incidence of acute and severe valve dysfunction due to pannus overgrowth in this study sample may appear high, but is comparable with those of two other previous studies, one on St. Jude aortic valve prostheses and the other comprising a mixed group of aortic, mitral, and tricuspid prosthetic valve prostheses[6,117]. Contrary to this, two large follow-up studies on the Medtronic-Hall valve in the aortic position reported no (0/736 patients) or an extremely low incidence (1/816 patients) of pannus-related valve dysfunction[113,118]. However, both latter publications focused on all-cause mortality after valve implantation and its predictors in cohorts comparable to those of this study. The low incidence of pannus reported in these studies may reflect the lack of recognition of pannus complications during follow-up. The high incidence of pannus observed in this study may be due to a high degree of recognition, confirmation by either operation or autopsy and due to the regional affiliation of patients to Haukeland University Hospital.

The time range between the primary operation and valve prosthesis dysfunction was wide, but similar to that of Vitale et al.[119]. They reported pannus obstruction ranging from some days to 12 years postoperatively in their study on pathologic findings of obstructed mitral prosthetic valves. Kondruweit et al. reported a case of valve obstruction due to pannus six months after surgery[116]. Thus, it is clinically important to consider pannus as a cause of acute dysfunction, irrespective of time since the valve prosthesis implantation (Fig. 10).

Suboptimal blood flow patterns and shear stress in the left ventricular outflow tract, high transvalvular pressure gradients, and incorrect valve orientation are all factors that may contribute to fibrous tissue overgrowth[120]. In the series of this study all patients had pannus affection of the minor orifice (Fig. 4 and 13). Speculations if

the minor orifice creates predisposing blood flow patterns or if the septum is more prone to pannus formation may be confounded by the patient selection. If septal hypertrophy had been a key factor for pannus formation, a higher incidence of readmission due to pannus obstruction would be expected since the predominant orientation at implantation was with the minor orifice facing the septum.

5.4.2 Limitations

The limitations of our study are primarily those related to its retrospective design. Haukeland University Hospital is the only hospital in western Norway equipped with a cardiothoracic surgical unit. All patients in this area requiring urgent or emergency cardiothoracic surgical treatment are transferred here. However, there is still a small chance that patients with acute prosthetic valve obstruction may have been taken to other hospitals or may have died suddenly. Thus, the 2.3 % incidence of acute and severe valve dysfunction due to pannus may be underestimated when the country is considered as a whole.

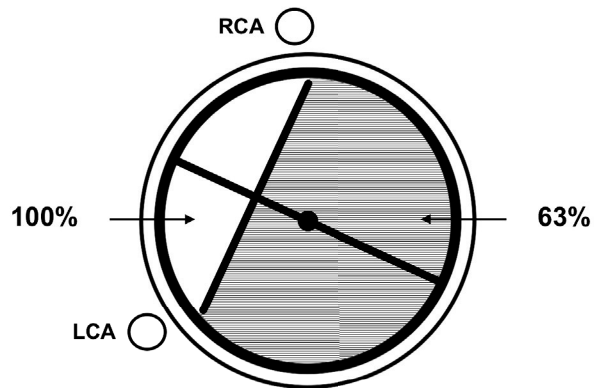

Figure 13. Localization of pannus in obstructed Medtronic-Hall aortic valve prostheses.

Pannus obstructed the minor valve orifice in 100% of the cases, while 63% had affection of the major orifice as well. LCA=left coronary artery; RCA=right coronary artery.

6. Conclusions

- Heparin coated vascular grafts improved patency and recruited less intimal hyperplasia compared to control uncoated vascular grafts at six months in a sheep model (**Paper I**).

- Proliferation and migration were inhibited by heparin only at higher concentrations due to increased MAPK-ERK phosphorylation at lower concentrations (**Paper II**).

- MAPK-ERK phosphorylation was influenced by heparin through both stimulatory and inhibitory pathways (**Paper II**).

- Co-culturing endothelial and vascular smooth muscle cells did not produce results different from the monocultures (**Paper II**).

- Enoxaparin and dalteparin did not inhibit cell proliferation at any concentration and migration was only slightly reduced at higher concentrations (**Paper III**).

- Proliferative and migratory effects of the two low-molecular weight heparins were different compared to heparin. The effects corresponded to the MAPK-ERK activation, suggesting different mechanisms of action (**Paper III**).

- 2.3 % of patients having received a Medtronic-Hall aortic valve prosthesis had been readmitted due to acute obstruction caused by pannus, resulting in an annual incidence of 0.7 per 1000 (**Paper IV**).

- Females and younger patients are at higher risk for pannus development, while valve size was not an independent risk factor (**Paper IV**).

7. Future Perspectives

The new technique for isolating and culturing ECs and VSMCs from blood vessel biopsies developed in conjunction with Paper II and III can be used in a variety of future experiments.

Testing the proposed hypothesis of antagonistic pathways controlling the IH response by blocking the FGF-pathway while simultaneously inhibiting mTOR with rapamycin and stimulating MPK-1, could yield therapeutically important results (Fig. 3).

The biobank currently contains cells isolated from the aorta. With a future expansion of the biobank, cultures from different segments of the vascular system can be isolated and compared with regard to all characteristica.

An interindividual difference of response to heparins was observed. Receptor mapping, proteomic and genetic studies may contribute to answer why there is a difference.

The co-culture method used generates *in vitro* organotypic capillary-like structures comprising basement membrane and pericyte enveloped three dimensional endothelial tubular structures resembling the *in vivo* organization[92]. Observing growth rate and pattern of co-cultures by time-lapse video and electron scanning microscope onto different types of graft material may provide novel and valuable knowledge about neointimal formation.

hFGF concentration at sites of iatrogenic injury is not known. Testing the MAPK-ERK response to heparin at other hFGF concentrations than 0 and 100 ng/mL may provide a broader theoretical basis of signalling responses in VSMCs (Fig. 9).

Pannus ought to be further characterized with regard to the histopathologic structure.

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