TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Genome-wide expression analysis of human bypass grafts with different degrees of atherosclerosis: Identification of an anti-apoptotic network and validation of candidate genes in *in vitro* and *ex vivo* systems.

Sara Katharina Dummler

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. M. Rychlik

Prüfer der Dissertation:

1. Univ.-Prof. Dr. J. J. Hauner
2. Priv.-Doz. Dr. H. Lahm, Ludwig-Maximilians-Universität München

Die Dissertation wurde am 13.11.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 07.02.2013 angenommen.

Publications Included in the Thesis

I. Krane M*, Dummler S*, Dressen M, Hauner H, Hoffmann M, Haller D, Heller K, Wildhirt S, Voss B, Grammer J, Lahm H, Lange R, Bauernschmitt R. Identification of an up-regulated anti-apoptotic network in the internal thoracic artery. Int J Cardiol. 2011 Jun 2;149(2):221-6. Epub 2010 Mar 6; * equally distribution

II. Dummler S*, Eichhorn S*, Tesche C, Schreiber U, Voss B, Deutsch MA, Hauner H, Lahm H, Lange R, Krane M. Pulsatile *ex vivo* perfusion of human saphenous vein grafts under controlled pressure conditions increases MMP-2 expression. Biomed Eng Online. 2011 Jul 21;10:62. * equally distribution

The main ideas and results of these papers are summarized in sections 4.1 to 4.2, respectively.

Other Publications

Krane M, Dummler S, Dressen M, Bauernschmitt R, Lange R. Bioengineering von Herzklappen. Kardiologie up2date 2008;4(4):276-280.

Published Abstracts

Dummler S, Krane M, Dressen M, Lahm H, Tesche C, Hauner H, Wildhirt S, Voss B, Badiu CC, Lange R, Bauernschmitt R. (2009) P197: Antiapoptotic Network May Contribute to Atherosclerotic Resistance of the Internal Thoracic Artery. *Arteriosclerosis, Thrombosis, and Vascular Biology Annual Confrence 2009*. ATVB 2009;29:e46.

Poster Presentations

Krane M, Dummler S, Dressen M, Hoffmann M, Heller K, Hauner H, Lahm H, Tesche C, Lange R, Bauernschmitt R. Up-regulation of the anti-apoptotic gene MCL-1 in the atherosclerosis-resistant internal thoracic artery. Annual congress of the European Society of Cardiology (ESC) 2009, Barcelona, Spain.

Dummler S, Krane M, Dressen M, Lahm H, Tesche C, Hauner H, Wildhirt S, Voss B, Badiu CC, Lange R, Bauernschmitt R. Antiapoptotic Network May Contribute to Atherosclerotic Resistance of the Internal Thoracic Artery. Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference 2009, Washington, D.C, USA

Dummler S, Dressen M, Krane M, Wildhirt S, Voss B, Badiu CC, Lange R, Bauernschmitt R. Global gene expression analysis reveals specific patterns of an anti-apoptotic pathway in the atherosclerotic resistant internal thoracic artery.
38th Annual Meeting of the German Society for Thoracic and Cardiovascular Surgery 2009, Stuttgart, Germany

Awards

Krane M, Dummler S, Dreßen M, Wildhirt S, Voss B, Badiu C C, Lange R, Bauernschmitt R. Gene Expression Profiling of the Atherosclerotic Resistant Internal Thoracic Artery Compared to the Atherosclerotic Prone Radial Artery. Award for the best poster at the 3rd German Atherosclerosis Congress 2008, Mannheim, Germany

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I Abstract

Coronary artery disease (CAD) which is directly linked to atherosclerosis is the most severe cause of death in the western world. Coronary artery bypass graft (CABG) surgery is the standard procedure in treatment of advanced CAD. To date graft patency rates remain far from being satisfactory and extension is, therefore, a major goal. As the atherosclerosis-resistant internal thoracic artery (ITA) has the longest patency rate, it is routinely used for CABG. The radial artery (RA) is known as an atherosclerosis-prone vessel with early atherosclerotic signs at time of surgery and minor patency rates. Nevertheless, despite rather unfavorable patency rates the use of the RA became an alternative to saphenous vein (SV) grafts with the lowest patency rate. Harvesting of the vein grafts and the following exposure to an arterial environment with higher flow rates and pressure profiles results in damage of the endothelial cell layer at an early stage which leads to graft thrombosis and the development of intimal hyperplasia (IH).

The purpose of this work was to compare the gene expression profile of the arteries and SV grafts in order to identify molecular markers being involved in early atherogenesis or intimal hyperplasia. I was able to identify anti-apoptotic candidate genes being up-regulated primarily in the intima of the atherosclerosis-resistant ITA compared to the atherosclerosis-prone RA. The results reveal a potential protective mechanism against atherogenic wall changes in mammary arteries. In addition, I was able to establish a new *ex vivo* perfusion system which enabled me to reliably measure molecular alterations after exposure of vessels to arterial conditions, which may be linked to the development of IH. As a proof of principle the gene expression of MMP-2, a gelatinase, which has been shown to play a central role in SMC migration and matrix degeneration and, therefore, atherosclerotic remodeling in the vessel wall, was shown to be strongly up-regulated in arterially perfused human saphenous vein grafts (HSVGs) in our system.

My work helps to more precisely understand the molecular mechanisms leading to an early failure in vein graft as well as helping to protect arterial bypass grafts against developing atherosclerotic wall changes. My results may be helpful to better understand the molecular mechanisms and therefore to elongate bypass graft patency rates in the future.

II Zusammenfassung

Koronare Herzerkrankungen (KHK) stehen in direktem Zusammenhang mit der Entstehung von Arteriosklerose und zählen zu den häufigsten Todesursachen in der westlichen Welt. Die koronare Bypasschirurgie ist das chirurgische Standardverfahren bei der Behandlung von KHK. Bis heute sind die Verschlussraten der Bypass-Gefäße allerdings alles andere als zufriedenstellend. Eine Erhöhung der Offenheitsraten ist daher ein bedeutendes Ziel. Die „Arteriosklerose-resistente“ *Arteria thoracica interna* (ITA) weist die höchste Offenheitsrate auf und wird deshalb als Standard-Gefäß bei Bypass-Operationen verwendet. Die *Arteria radialis* (RA) ist als „Arteriosklerose-anfälliges“ Gefäß bekannt und zeigt häufig bereits vor der Bypass-Operation Zeichen früher Arterioskleroseentwicklung, wird aber, bedingt durch ihre höhere Offenheitsrate, als Alternative zur *Vena saphena magna* (SV) immer öfter verwendet. Die SV weißt die geringste Offenheitsrate auf, ist aber länger und leichter zu entnehmen als die RA.

Die Entnahme der Venen und das anschließende Einbringen des Gefäßes in das arterielle Hochdruck-System mit weit höheren Druck- und Flussgeschwindigkeiten resultiert in der frühen Verletzung des einschichtigen Endothels des Blutgefäßes und führt damit zu Thrombosen und der Entwicklung intimaler Hyperplasie.

Das Ziel dieser Arbeit bestand darin, Gen-Expressionsprofile der Arterien und Venen zu vergleichen, um letztendlich Biomarker identifizieren zu können, die möglicherweise Aufschluss über die Entwicklung von früher Arteriosklerose oder intimaler Hyperplasie liefern. Beim Vergleich der beiden Arterien ITA und RA konnte ich ein anti-apoptotisches Kandidatengen-Netzwerk in der intimalen Schicht der „Arteriosklerose-resistenten“ ITA im Vergleich zur „Arteriosklerose-anfälligen“ RA identifizieren.

Die Ergebnisse der Studie lassen vermuten, dass sich die ITA durch die Expression der anti-apoptotischen Gene gegen atherogene Wandveränderungen schützen kann.

Zusätzlich konnte ich ein neues *ex vivo* Perfusionssystem etablieren und damit diverse Biomarker in Venen identifizieren, die nach Perfusion unter arteriellen Hochdruckbedingungen stark hochreguliert waren und mit der Entwicklung von intimaler Hyperplasie in Zusammenhang stehen könnten. Zum Nachweis dafür konnte gezeigt werden, dass die Expression der Gelatinase MMP-2, die bei der

Degeneration von extrazellulären Matrixproteinen und damit bei arteriosklerotischen Umbauvorgängen in der Gefäßwand sowie bei der Migration und Proliferation von glatten Muskelzellen eine zentrale Rolle spielt, in arteriell perfundierten Venen stark erhöht war. Dadurch kann meine Arbeit helfen, die molekularen Mechanismen, die zu einem frühen Verschluss der venösen Bypass-Grafts führen oder die arteriellen Gefäße vor der Entstehung von Arteriosklerose schützen, zu identifizieren. Meine Ergebnisse können somit durch das bessere Verständnis der molekularen Mechanismen dazu beitragen, in Zukunft die Offenheitsraten und damit die Haltbarkeit der Bypass-Gefäße zu verlängern.

III LIST OF ABBREVIATIONS

$ApoE^{-/-}$	apolipoprotein E knockout
BAX	Bcl-2-associated protein X
BCL-2	B-cell lymphoma 2
BSA	bovine serum albumin
CABG	Coronary arterial bypass graft
CAD	Coronary Artery Disease
CCL2	Chemokine (C-C motif) ligand 2
CD31	cluster of differentiation 31
CDKN1A	cyclin-dependent kinase inhibitor 1A, also called p21
CDKN2A	cyclin-dependent kinase inhibitor 2A, also called p16
CVD	Cardiovascular Diseases
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
EC	endothelial cell
FC	fibroblast cell
FCS	fetal calf serum
FFPE	Formalin-fixed and paraffin embedded
HSVG	human saphenous vein graft
ICAM-1	Intercellular Adhesion Molecule 1
IER3	immediate early response gene 3
IGF-1	Insulin-like-Growth Factor 1
IH	intimal hyperplasia
IL	Interleukin
IMR	intima-to-media ratio
ITA	internal thoracic artery
ITI	intimal thickness index
LDL	low density lipoprotein
LMD	Laser Microdissection
%LN	percent of luminal narrowing
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor

MCL-1	myeloid cell leukemia sequence 1
MCP-1	monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NIH	neointimal hyperplasia
NO	nitric oxide
oxLDL	oxidized low density lipoprotein
PAD	peripheral artery disease
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PIM1	Proto-oncogene serine/threonine-protein kinase
PKCδ	Protein kinase C gamma
PROK2	Prokineticin 2
qRT-PCR	quantitative real-time polymerase chain reaction
RA	radial artery
ROS	reactive oxygen species
SAM	Significance Analysis of Microarrays
SMC	Smooth muscle cell
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducer and activator of transcription
SV	saphenous vein
TNC	Tenascin C
TNF-α	tumor necrosis factor alpha
VEGFA	vascular endothelial growth factor A
VSMC	vascular smooth muscle cell
WHO	World Health Organization

1. Introduction

1.1 Background

According to the World Health Organization (WHO) cardiovascular diseases (CVD) with over 17 million deaths in 2008 are the leading cause of death for noncommunicable diseases worldwide. By 2030, almost 23.6 million people will die from CVDs mainly from heart disease and stroke (WHO, 2010). Coronary artery disease (CAD) is a CVD, which is directly linked to atherosclerosis and it is the most frequent cause of death in the Western World (Naghavi *et al.*, 2003).
Several risk factors for atherogenesis like hypercholesterolemia, diabetes, cigarette smoking and hypertension have been identified in the past years (Borden *et al.*, 2009; Hall & Lorenc, 2010). Of those, hypertension seems to be one of the most critical factors (Nguyen, 2010; Motwani, 1998; Favaloro, 1968). Similar to aging it is associated with structural remodeling and stiffening of large arteries, leading to CAD and atherosclerosis (Motwani, 1998), which in turn can cause myocardial infarction and stroke. More than $140 billion are spent each year for the diagnosis and management of CAD only by the United States (Heston, 2011). Coronary arterial bypass graft (CABG) surgery is the standard procedure for treatment of advanced CAD with annual expenses of more than $26 billion in the United States (Cundiff, 2002). Nevertheless, graft patency rates remain far from being satisfactory at present. Long-term patency of the bypass graft is the major factor determining survival in patients after CABG surgery and its extension, therefore, remains a major goal.

1.2 Coronary artery bypass graft (CABG) surgery

Despite the development of different percutaneous coronary interventions CABG is still the gold standard for the treatment of multivessel CAD. In modern bypass surgery different vessels are taken for revascularization in patients with CAD. The internal thoracic artery (ITA) is the first choice of bypass grafts, since the “exceptional” long-term patency of the ITAs was documented by Lytle et al. already in the 1980s

(Lytle *et al.*, 1980; Lajos *et al.*, 2007). Since then the attention of the surgeons turned towards the usage of bilateral ITAs and other arterial bypass grafts like the radial artery (RA). The RA was first used in the 1970s by Carpentier (Carpentier *et al.*,1973), but soon was put aside because of the poor patency rates. Acar reintroduced the RA as a bypass graft 20 years later by showing good long-term results (Acar *et al.*, 1992), improvement in the technique of harvesting and the use of calcium channel blockers (Kobayashi, 2009). Recently, the RA became a reasonable alternative to saphenous vein (SV) grafts and is particularly used in younger patients (age<70) (Wildhirt *et al.*, 2006). Patency rates of the RA have improved considerably since the early efforts. Long-term (10 year) patency rates of 89 to 94% have been reported for RA grafts. SV grafts showed patency rates of 74 to 92% and the left ITA even rates of 97 to 99% (Passati *et al.*, 2003; Cohen *et al.*, 2001). There is a trend towards a complete arterial revascularization by replacing the SV graft with the RA. However, one major point when using RAs may be advanced atherosclerosis at the time of surgery (5% to 10% incidence) (Chowdhury *et al.*, 2004; Oshima *et al.*, 2005; Manabe & Sunamori, 2006) which may represent an increased risk for early stenosis and occlusion of the graft (Khot *et al.*, 2004).

Using the great saphenous vein as bypass graft is also a standard procedure in the treatment of advanced CAD. The human SV is the first (Favaloro *et al.*, 1969) and one of the most commonly used conduits for CABG today. In contrast to the ITA and the RA it is easy to use as a bypass graft because of its large diameter and wall characteristics, its length and the relatively easy harvesting procedure (Sabik, 2011). Yet, still a major problem remains with vein graft occlusion after surgery. In the first month after bypass surgery 10 to 15 % of the HSVGs occlude and an additional 25% within the first year. Mehta and colleagues even reported a HSVG failure of 40 to 50% during the first year (Mehta *et al.*, 2011). Ten years after surgery about 50% of the veins have failed and of those who remain patent 25% have been severely stenosed (Campeau *et al.*, 1984; Dashwood, 2009; Fitzgibbon *et al.*, 1996; Grondin *et al.*, 1984; Kloppenburg *et al.*, 2009). Harvesting of the veins and the following exposure to an arterial environment with much higher flow rates and pressure profiles rapidly leads to damage of the endothelial cell (EC) layer. This in turn may lead to graft thrombosis and the development of intimal hyperplasia (IH). In contrast to the HSVG, the RA is adapted to higher arterial pressures. Nevertheless, the RA is also prone to develop intimal thickening and atherosclerosis.

1.3 Vascular structure of ITA, RA and SVG

Despite the common basic functions of arteries and veins in the transportation of blood there are many differences in their morphology and physiology (Figure 1). Both vessel types consist of three distinct layers: the intima, the media and the adventitia. The three layers are separated from each other by the internal and external elastic laminae. The intima is composed of a confluent monolayer of ECs which form the endothelium. The ECs are arranged longitudinally, are orientated in the direction of blood flow and they assure hemocompatibility and anti-thrombogenicity of the vessel. The media consists of smooth muscle cells (SMC) aligned concentrically along with collagen, elastin fibers and proteoglycans. This layer is the major component of muscular arteries like the RA. The media and the elastic layer of veins consist of a limited number of cells. Therefore, a venous vessel wall is typically much thinner than an arterial one (Figure 1 A) (Muto *et al.*, 2010). The adventitia, as the third layer, is mainly composed of collagen and fibroblasts (FC), both of which are arranged longitudinally. The function of this layer is basically the same in all types of vessels. It provides anchorage within connective tissues and maintains the nutrient support and vascularization to the internal layers of the vessel.

In addition to the three layers, veins have a very special structure in their lumen, the venous valve. In contrast to the thick muscular layer in arteries which supports the blood transport via peristaltic effects the venous valves are responsible for the blood return to the heart by avoiding blood reflux under physiological conditions (Muto *et al.*, 2010).

The ITA differs from the RA in some morphological features (Figure 1 B). The RA as a muscular artery has a thicker media with SMCs arranged in several tight layers (Acar *et al.*, 1991; Gansera *et al.*, 2004). Furthermore, the elastic laminae of the RA are monolayers with multiple fenestrations while the ITA lacks those and forms a multilayer (Kaufer *et al.*, 1997). Those well-developed elastic laminae prevent the invasion of SMCs and protect the vascular wall from atherosclerotic changes in the ITA. The endothelial layer is able to produce plenty of nitric oxide (NO) and thus can regulate the vascular tonus according to the blood flow demand (Manabe &Sunamori, 2006).

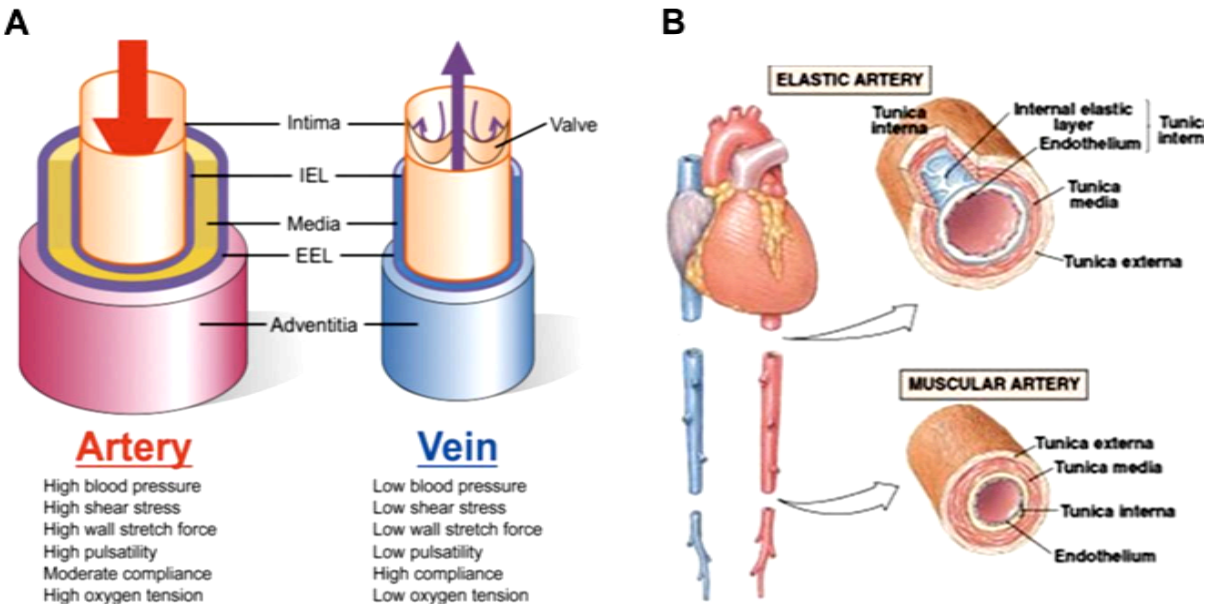

Figure 1: Morphology and physiology of arteries and veins. A) Morphological and physiological features of arteries and veins. IEL internal elastic lamina; EEL external elastic lamina. B) Morphological features of elastic (ITA) and muscular arteries (RA). Source: Muto et al. Circ J 2010 (Muto *et al.*,2010).

The physiological environments of arteries and veins differ in their pressure and flow state. Arteries are exposed to high pressure and flow conditions (~60-140 mmHg, shear stress of 10-70 dyne/cm^2) during the cardiac cycle, whereas the venous wall is usually exposed to low pressure conditions (~5-10 mmHg), low flow state and a magnitude of shear stress in the range of 1- 6 dyne/cm^2 (Malek *et al.*, 1998; Smith *et al.*, 2006).

1.4 Atherosclerosis and Intimal Hyperplasia

1.4.1 Atherosclerosis

Atherosclerosis is a disease of the arterial wall that occurs at susceptible sites in the major conduit arteries (Insull, 2009). It is initiated by lipid accumulation in the intima, lipid oxidation, and modification, which provoke chronic inflammation, ultimately causing stenosis with potentially lethal distal ischemia or trigger thrombotic occlusion of major conduit arteries to the heart, brain, legs, and other organs (Insull, 2009).

The idea of inflammation-based mechanisms in atherogenesis was first described by Rudolf Virchow in the mid-19th century (Lamon & Hajjar, 2008). Since then, many hypotheses have been proposed to include the role of inflammation in atherosclerosis (Kádár & Glasz, 2001). One of the best known is the “response to injury” model of Russell Ross, established in the 90ies of the last century (Ross, 1993 and 1999). In this model, injury to the endothelium due to mechanical trauma, shear stress, infection, and/or an increase in reactive oxygen species (ROS) is believed to be the initial trigger for a local inflammatory response. The injured endothelium becomes permeable to lipoproteins and other plasma constituents which are mediated by several factors like NO, platelet-derived growth factor (PDGF), endothelin or the up-regulation of leukocyte and endothelial adhesion molecules. The low density lipoprotein (LDL) reacts with proteins and free radicals in the extracellular matrix forming an oxidized complex. The oxidized LDL (oxLDL) particles trigger the immune system by attracting monocytes to the inflammation site and mediate the migration of the monocytes into the arterial wall. This process is also mediated by cell adhesion molecules like monocyte chemoattractant protein 1 (MCP-1, also called CCL2), interleukin-8, PDGF or macrophage colony-stimulating factor (M-CSF). The monocytes start to proliferate and differentiate into macrophages which in turn secrete cytokines stimulating the migration of further monocytes and other circulating leucocytes to the injured wall. Macrophages start to express scavenger receptors that recognize and bind to the oxLDL and the rapid up-take of lipids lead to foam cell formation. The lipid-laden foam cells together with T-lymphocytes form fatty streaks which are later joined by migrated SMCs from the medial layer. The SMCs produce extracellular matrix (ECM) molecules, including interstitial collagen and elastin, and form a fibrous cap that covers the plaque. This cap typically overlies a collection of macrophage-derived foam cells, some of which die and release lipids that accumulate extracellularly. The inefficient clearance of dead cells — a process known as efferocytosis — can promote the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque (Libby *et al.*, 2011). Thinning of the fibrous cap due to the continuing influx and activation of macrophages which release matrix metalloproteinases (MMPs) and other proteolytic enzymes, can lead to rupture or ulceration. The rupture of the fibrous cap maintains exposure of plaque contents to the blood flow and therefore

can rapidly lead to thrombus formation and occlusion of the vessel (Figure 2) (Ross, 1999; Libby *et al.*, 2011; Kádár & Glasz, 2001).

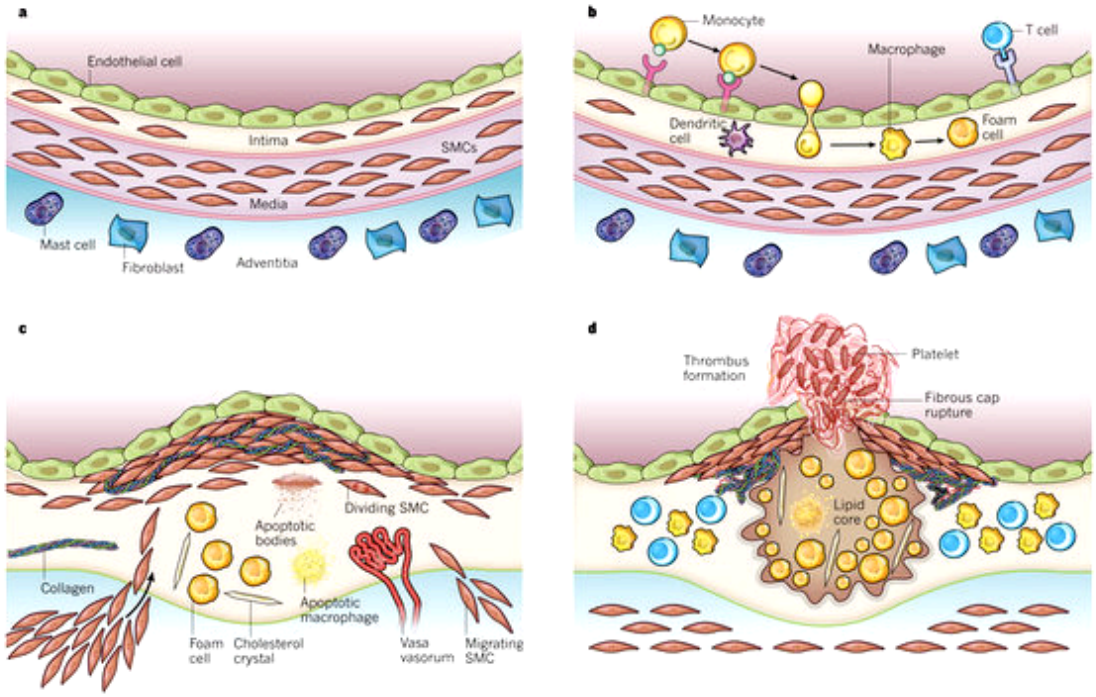

Figure 2: Stages in the development of atherosclerosis. a) normal artery wall. b) initial steps of atherosclerosis including adhesion and migration of blood leukocytes, transformation into macrophages and development of foam cells by lipid uptake. c) Lesion progression involving migration and proliferation of SMCs, synthesis of extracellular matrix and accumulation of extracellular lipid derived from dead or dying cells forming the necrotic core. d) Thrombus formation, rupture of the fibrous cap and occlusion of the artery. Source: Libby *et al*., Nature May 2011;473.

1.4.2 Intimal Hyperplasia

Intimal hyperplasia (IH) describes the thickening of the intimal layer of a blood vessel and is a general response of the grafted vessel to injury after harvesting in CABG surgery. IH is a process which includes cell proliferation and differentiation and occurs as a consequence of physiological stimuli, constituting an attempt by the tissue to maintain normal conditions of flow, wall tension, or both (Ruengsakulrach *et al.*, 1999). Yet, excessive formation of IH typically precedes atherosclerosis and is an important reason of late bypasses graft failure. The great majority of RA conduits show signs of preexisting intimal hyperplasia mostly affecting the distal portion whereas nearly no wall thickening can be detected in ITAs (Chowdhury *et al.*, 2004; Reddy *et al.*, 2011).

IH is characterized by proliferation of SMCs and their migration through the internal elastic lamina into the sub-intimal layer with production of ECM proteins (Rey *et al.*, 2004) and is the principal mechanism of graft failure between 1 and 12 months after implantation (Ranjzad *et al.*, 2009). IH is also referred to as neointimal hyperplasia (NIH) which occurs as a consequence of vessel wall injury particularly after CABG surgery or balloon injury and is a major problem especially in grafted veins. Harvesting of the veins and the following exposure to an arterial environment with much higher flow rates and pressure profiles results in damage of the EC layer at an early stage which in turn leads to graft thrombosis and the development of NIH. Under physiological conditions human SVs are exposed to low pressure conditions, a nonpulsatile flow and a low shear stress (Malek *et al.*, 1999). After grafting and implantation into the coronary artery system the graft must support the higher arterial pressure conditions, a pulsatile flow and a higher shear stress during the cardiac cycle (Malek *et al.*, 1999; Smith *et al.*, 2006). The endothelial dysfunction leading to NIH and eventually atherosclerosis increases the permeability for macromolecules (i.e. lipoproteins), and the expression of chemotactic and adhesion molecules (i.e. Intercellular Adhesion Molecule 1, ICAM-1 or E-selectin). The recruitment of monocytes and macrophages into the intima is stimulated and the intimal injury results in platelet adhesion to the intimal surface and stimulation of SMCs to proliferate and migrate into the intimal layer within the first week after grafting (Sabik, 2011; Motwani & Topol, 1998; Gusic *et al.*, 2005).

The vein graft –in contrast to others- is able to adapt to the arterial environment after surgery by this intimal thickening process which is also known as “arterialization” (Figure 3). The vascular wall remodeling is thought to be a necessary response for the vein graft to adapt to the higher wall shear stress and other flow patterns. Nevertheless, at least 20-50% of the vein grafts develop thrombosis and fail due to these vascular remodeling processes. The vein graft adaptions to the arterial environment show a pattern similar to that of the post-arterial injury response. However, it is likely that vein graft adaption occurs via specific and distinct molecular mechanisms which are not clearly understood to date (Muto *et al.*, 2010).

The development of atherosclerosis *in vivo* is a lifelong process which usually starts in early youth with fatty streak formation. IH and NIH occur in particular regions of the blood circulation *in vivo* and appear early in bypass grafts after CABG surgery.

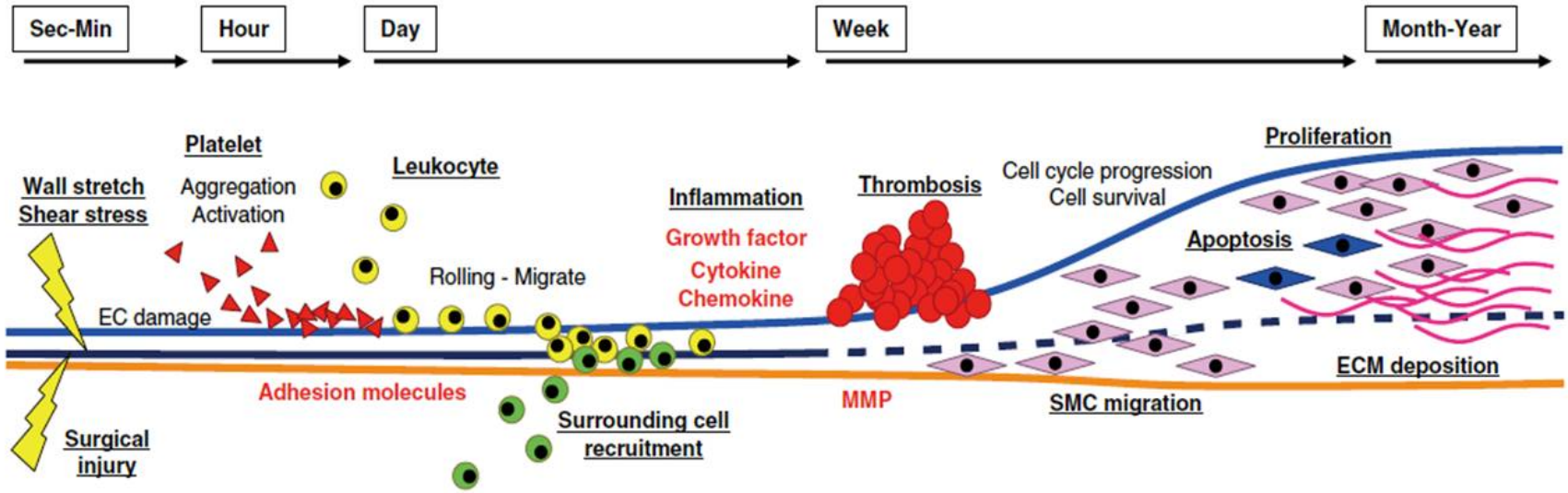

Figure 3: Time course of vein graft neointimal formation; Source: Muto *et al.*, Circ J 2010,74:1501–1512.

The metabolic reactions are very similar and IH often results in atherosclerosis. Therefore, the intimal wall thickening and examination of the molecular biomarkers which are linked to its progression are suitable for studying atherogenesis in human vessels.

1.5 Gene expression analysis

After the draft human genome sequence was published in February 2001 (Lander *et al.*, 2001; Venter *et al.*, 2001) many new research techniques evolved. Disease-related genes could be discovered in large scale genome studies shifting from a candidate gene approach to systemic genetic approaches, in which the full complexity of disease processes could be investigated (Tegner *et al.*, 2007).
Whole transciptome measurements are evaluated by the microarray technique which allows concomitant screens of gene activity of tens of thousands of genes. Microarrays may discover entirely novel and unexpected functional roles of genes due to the “global” approach. Today a multitude of array types like “whole genome-” (Figure 4), “theme-specific-” or “customized” arrays are available (Tuomisto & Binder, 2005). The vast amount of DNA microarray data can be organized by clustering algorithms that classify functionally associated genes in terms of co-expression. Biologically relevant information is extracted using pathway and Gene Ontology (GO) analysis to generate biological pathways (King *et al.*, 2005).

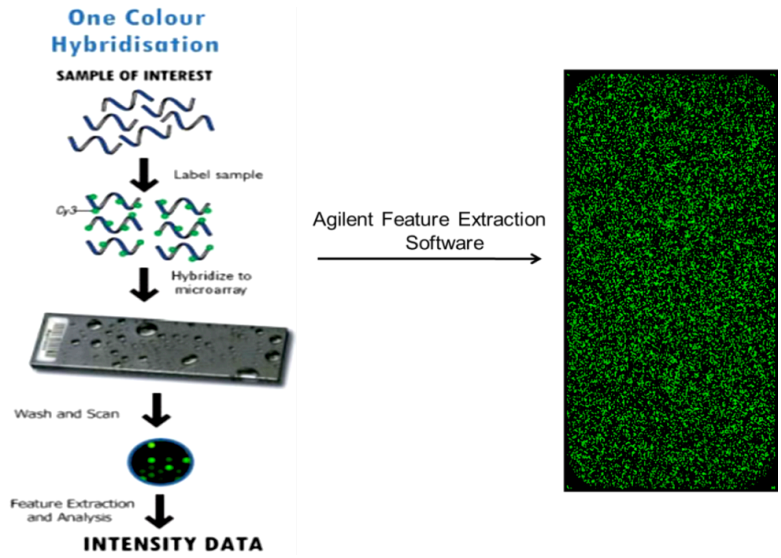

Figure 4: Schematic representation of an Agilent Whole Human Genome 4x44k microarray one colour set-up. Source: Agilent Technologies.

Several efforts have been made to study large-scale gene expression in human atherosclerosis. A variety of microarray studies with EC or SMC cultures have been made to gain insight into the development of atherosclerosis (Chen *et al.*, 2011). Still genome-wide information obtained from various cell culture experiments may not accurately reflect the molecular events occurring *in vivo*. Therefore, animal models and most notably knock-out mouse models were used to identify molecular mechanisms in atherosclerosis. Two large clusters or functional gene groups of inflammation and proteolysis for instance were identified in a microarray assay of atherosclerotic aortic arches from apolipoprotein E knockout ($ApoE^{-/-}$) mice (Lutgens *et al.*, 2005; Gräbner *et al.*, 2009). Yuan et al. reported the involvement of calcium-signaling in the inflammatory process of atherosclerosis by studying microarray analysis of gene expression in $ApoE^{-/-}$ mouse aortas (Yuan *et al.*, 2009). Inflammation and immune signals could also be observed in human atherosclerotic lesions by comparing gene expression in normal and atherosclerotic arteries (Martinet *et al.*, 2002; McCaffrey *et al.*, 2000; King *et al.*, 2005). The results of those studies have further suggested that inflammation and oxidative stress play important roles in the development and progression of atherosclerosis (Chen *et al.*, 2011).
The understanding of complex biological pathways due to large genome-wide expression analyses may give insight into function and interaction of genes and proteins involved in the pathogenesis of IH and atherosclerosis: They may, therefore, help to unravel new diagnostic markers, therapeutic targets and treatments of various diseases from the bench to the bedside.

1.6 *In vivo*, *in vitro* and *ex vivo* models of Intimal Hyperplasia and early atherosclerosis

Various studies exist about the development of IH and atherosclerosis in different models. *In vivo* experiments are conducted predominantly with mouse models. The transgenic $ApoE^{-/-}$ mouse (Zhang *et al.*, 1992) is one of the most commonly used animal models. ApoE is involved in lipoprotein uptake by the LDL receptor in blood circulation. $ApoE^{-/-}$ mice develop hypercholesterolemia and atherosclerotic lesions when fed with a high cholesterol diet. To date, numerous studies have been performed with ApoE knockout mice to study the development of atherosclerosis or

IH (Imaizumi, 2011; Kolovou *et al.*, 2008; Feig *et al.*, 2009; Bond & Jackson, 2011; Murphy *et al.*, 2011; Zhu *et al.*, 2001; Leidenfrost *et al.*, 2003; Matter *et al.*, 2006; Sasaki *et al.*, 2006; Ni *et al.*, 2009).

In vitro experiments often use primary human or animal cell cultures or established cell lines to study atherogenesis. Nowadays, multiple cells are available from different types of human or animal vessels and tissues. One major part of atherosclerosis studies is focused on blood flow and laminar shear stress (Yoshizumi *et al.*, 2003; Surapisitchat *et al.*, 2001; Li *et al.*, 2008; Ohno *et al.*, 2005; Partridge *et al.*, 2007; Yoshisue *et al.*, 2002), which has emerged as an essential feature of atherogenesis. Maintenance of a physiological laminar shear stress is known to be athero-protective and crucial for normal vascular functioning. This includes the regulation of vascular tone as well as inhibition of proliferation, thrombosis and inflammation of the vessel wall. Disturbed or oscillatory flow conditions which occur close to arterial bifurcations and curvatures are associated with changes to endothelial gene expression, cytoskeletal arrangement, wound repair, leukocyte adhesion as well as atheroma formation (Cunningham & Gotlieb, 2005). Disturbed shear stress also influences vessel wall remodeling which can affect plaque vulnerability, stent restenosis and SMC IH in venous bypass grafts (Kwei *et al.*, 2004; Abeles *et al.*, 2006). Nevertheless, all cell culture systems studying atherogenesis to date are limited to basically one cell type and, therefore, do not allow the study of cell-cell interactions or the reenactment of the morphological and functional characteristics of the human vascular wall.

One way to better explore the *in vivo* situation in human material are *ex vivo* studies. Their great benefit is the ability to perform experiments and measurements which would not be possible in living subjects due to technical or ethical reasons. *Ex vivo* studies allow to measure physical, thermal, electrical, mechanical or optical tissue properties which are partly extreme and under life-threatening conditions. In addition, they are used to investigate new surgical procedures or imaging techniques. They are also widely used to study blood flow pattern, cell behavior and molecular mechanisms in regions with undisturbed laminar or disturbed oscillatory flow. *In vivo* studies to examine disturbed flow have been made with occlusion models, placement of arteriovenous fistula or ligation of large veins (Berceli *et al.*, 2004; Kwei *et al.*, 2004; Abeles *et al.*, 2006). Large veins from humans or animals are usually taken for *ex vivo* perfusion studies, though human samples are much less frequently used.

Several studies with *ex vivo* systems analyzed histomorphological changes, development of IH and gene expression of plasminogen activator inhibitor 1 (PAI-1) or MMPs in vein grafts after perfusion with different pressure profiles (Paroz *et al.*, 2004; Rey *et al.*, 2004; Patterson *et al.*, 2001; Galis, 2004; Mavromatis *et al.*, 2000). Others investigated vein remodeling under static or basic conditions (Wilson *et al.*, 1997; Porter *et al.*, 1996; Golledge *et al.*, 1997; Muluk *et al.*, 1998), the role of the mechanical environment during the remodeling process in higher developed perfusion systems (Gusic *et al.*, 2005; Saucy *et al.*, 2010) or gene expression or pathways linked to hypertension (Lauth *et al.*, 2000; Dashwood, 2009).

1.7 Apoptosis and atherosclerosis

Apoptosis has been identified as a prominent feature of advanced human atherosclerotic plaques, affecting the cellularity and integrity of the plaques.
The process of apoptosis can be mediated via two distinct mechanisms: the death ligand- and the mitochondria-mediated pathways. Each is regulated by various environmental and endogenous factors such as the inflammatory process (Littlewood, 2003). Apoptosis plays a dual role for SMCs in atherosclerosis: in early stages, apoptosis of SMCs and inflammatory cells may delay the atherosclerotic process, while in late stages it may render the atherosclerotic plaque from vulnerable to rupture (Katsiki *et al.*, 2010). Excessive accumulation of SMCs in intima and media of atherosclerotic lesions involve both an abnormal proliferation and reduced apoptosis of these cells. Alteration of the balance between proliferation and apoptosis of SMCs is thought to play an important role in atherosclerosis formation and subsequent cardiovascular complications (Hsieh *et al.*, 2000; Clarke & Bennett, 2006).
Recent reports discuss the involvement of apoptotic mechanisms in the development of early atherogenesis (Karaflou *et al.*, 2008; Kockx & Herman, 2000; Martinet *et al.*, 2002; Martinet *et al.*, 2003). Throughout atherogenesis both SMCs and ECs undergo programmed cell death (Tabas, 2007), thus the apoptosis of ECs is one of the earliest events in the development of IH and atherosclerosis and leads to endothelial dysfunction. However, the general understanding of the apoptotic process in atherogenesis as well as the anti-apoptotic and athero-protective mechanisms in some arteries resistant to IH and atherosclerosis remain unclear at present.

2. Aim

The overall aim of this thesis was to identify differences in the gene expression profile of CABG bypass vessels which are potentially related to the development of atherosclerosis and/or intimal hyperplasia in the grafts. The differential expression of biomarkers in the arterial bypass grafts ITA and RA with variable grades of early atherosclerotic developments were analyzed by a genome-wide expression microarray and subsequent pathway analyses. The construction and establishment of a novel *ex vivo* perfusion system was used to study changes in gene expression of molecular markers being involved in the NIH of venous bypass grafts particularly under arterial perfusion conditions.

Specific aim of each paper

I. To identify genes and gene pathways which are related to differences in susceptibility of developing atherosclerosis in two different bypass grafts of the same patients
II. Design of a novel *ex vivo* perfusion system with standardized and strictly controlled hemodynamic parameters for the pulsatile and non-static perfusion of HSVGs to guarantee a reliable analysis of molecular parameters under different pressure conditions

3. Methods

3.1 Patient recruitment

From 2007 to 2011 more than 140 patients undergoing CABG surgery in the German Heart Center Munich were included into this study. All patients signed an informed consent for inclusion and the study was approved by the ethical committee at the Technische Universität München, Medical Faculty (file number 1588/06) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

3.2 Tissue collection

Tissue samples of more than 80 ITA or RA samples from the same or different patients and samples of nonvaricose HSVGs from 61 patients were obtained during CABG surgery. The endoscopically harvested RAs and veins were kept in autologous blood at room temperature until implant. Parts of the HSVGs were immediately transferred in ice-cold ringer solution on ice to the laboratory. ITA and RA samples were immediately divided into two parts in the operating room: one was fixed in 4% PBS-buffered formalin (pH 7.4) and processed for histology and morphometry, while the other part was frozen in liquid nitrogen and stored at -80°C for RNA analysis.

Vein grafts were similarly divided. One small piece was directly snap-frozen in liquid nitrogen and stored at -80°C until further use. This piece served as a reference to determine relative gene expression. A second piece was fixed in 4% PBS-buffered formalin (pH 7.4) for histology and morphometry. The largest part of the vein was mounted into the perfusion device of the *ex vivo* perfusion system (Dummler *et al.*, 2011).

3.3 Histology and Morphometry

Formalin-fixed and paraffin embedded (FFPE-) tissues from ITA, RA and HSVG samples were cut in sections of 3 and 4 µm, deparaffinized and stained with hematoxylin and eosin (HE). Tissue samples of ITA and RA from 50 patients could be processed for morphometric analysis. In this study, three morphometric parameters were used to describe the degree of intimal thickening (Table 1) (Wildhirt *et al.*, 2006). Histomorphometric data were analyzed using the Image-Pro® Software (Media Cybernetics, Inc., MD) and ImageJ (Open source, http://rsb.info.nih.gov/ij/).
To verify tissue viability of HSVGs in the *ex vivo* perfusion system a staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma, Munich, Germany) was performed. In the presence of metabolically active viable cells the yellow MTT is converted into a water-insoluble purple formazan product due to reduction by mitochondrial dehydrogenases and other cellular enzymes (Bernas & Dobrucki, 2002; Burunova *et al.*, 2010). MTT was stored as a stock solution (5 mg/ml in PBS) at -20°C. Short segments of veins (n=12) were incubated in MTT diluted in serum-free medium to 0.5 mg/ml for one hour at 37°C.

Table 1: Morphometric parameters

Morphometric parameters	
ITI	$\frac{intimal\ area}{medial\ area}$
IMR	$\frac{maximal\ width\ of\ intima}{maximal\ width\ of\ media}$
%LN	$\frac{100\ x\ intimal\ area}{internal\ elastic\ lamina}$

ITI: intimal thickness index; IMR: intima to media ratio; %LN: percent of luminal narrowing*; internal elastic lamina = Lumen + Intima*

3.4 Immunohistochemistry

Immunohistochemistry was performed on FFPE-sections of HSVGs. The sections were stained with the primary antibodies against CD31 (Santa Cruz Biotechnology, Heidelberg, Germany), Ki67 and Caspase3 (Zytomed Systems, Berlin, Germany).

CD31 is expressed on the surface of adult and embryonic endothelial cells (Righi *et al.*, 2003). Ki67 is a cellular marker for proliferation and is absent from resting cells (Scholzen & Gerdes, 2000), and Caspase 3 is a marker for cells that undergo apoptosis (Mazumder *et al.*, 2008). Sections were then incubated with an Avidin/Biotin detection system (Vectastain ABC-Kit Elite Universal; Biozol, Eching, Germany) and were visualized under a light microscope (Axiovert 200M, Zeiss, Oberkochen, Germany) (Table 2). Slides incubated without the primary antibody served as a negative control. Detailed immunohistochemical (IHC) methods can be found in supplementary table 1.

Table 2: Primary and secondary antibodies.

Antibody	**Species**	**Dilution**	**Company**
primary Antibody			
CD31	Mouse IgG1	1:30	Dako
Ki67	Mouse IgG1	1:100	Dako
Caspase 3	Rabbit IgG	1:100	Zytomed systems
secondary Antibody			
Vectastain ABC-Kit Elite Universal detection system Biotinylated Universal antibody	Anti-Mouse IgG/Rabbit IgG	1:50	Biozol
Dako EnVision™ FLEX /HRP detection reagent	Goat-anti-Mouse IgG	Unspecified	Dako

3.5 Gene expression studies

3.5.1 Whole Genome Microarray and Pathway analysis

After morphometrical analysis, paired ITA and RA samples of 6 patients were selected for microarray analysis. Total RNA was isolated using standard RNA extraction protocols (Trizol, Sigma-Aldrich, Steinheim, Germany). RNA quality was assessed by the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Böblingen, Germany) and was selected for the linear T7-based amplification (according to the manufacturer's protocol). The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene

Expression Hybridization Kit (Agilent Technologies). A Significance Analysis of Microarrays (SAM) was performed in order to identify genes which were consistently differentially regulated in all patients. The resulting discriminatory genes were provided in all clustered row-normalized heat maps. The list of genes significantly up- or down-regulated in the RA samples in comparison to the ITA samples were tested for a significant enrichment of annotation using the proprietary TreeRanker software (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). To identify the biological context of the discriminatory genes, pathway analysis was performed by using the PathwayArchitectTM software (Stratagene, La Jolla, CA) to generate molecular networks (Miltenyi Biotec GmbH).

3.5.2 Quantitative RT-PCR

Frozen tissue specimens were homogenized using the Precellys24 system (Peqlab, Erlangen, Germany) and total RNA was extracted using Trifast according to the manufacturer's protocol (Peqlab). All RNA preparations were digested with DNase I prior to cDNA synthesis using Omniscript RT kit (Qiagen, Hilden, Germany). Expression analyses of candidate genes of an anti-apoptotic pathway (Krane *et al.*, 2011) and of other pro-apoptotic and vascular genes were performed using a Light Cycler 1.5 (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green Kit (Qiagen) and BSA (0.5 mg/ml) in a final volume of 20µl. All primers (supplementary Table 2) were used in a final concentration of 0.5 µM. They amplify fragments between 66 and 189 bp, respectively. After an initial activation of *Taq* polymerase for 15 min at 95°C specific products were amplified during 40 cycles using the following conditions: 15 sec at 94°C (denaturation), 20 sec at 60°C (annealing) and 20 sec at 72°C (elongation). In each cycle the fluorescence was determined after an incubation of 5 sec at 78°C thereby avoiding the inclusion of low-melting products such as primer dimers in the measurement. The relative expression levels of the examined genes in individual samples were calculated in relation to the expression of GAPDH or ß-actin housekeeping genes. The calculation of these ratios allows the comparison of independent samples.

3.6 Laser microdissection

The Leica LMD6000 (Leica Microsystems, Wetzlar, Germany) was used according to the manufacturer's instructions. FFPE tissue samples of ITA and RA from four of the six patients were cut in 5-µm sections on a microtome and collected onto PET UV-absorbing membrane slides (RNAse free, Leica Microsystems). Sections were deparaffinized in a Leica ST5020 Multistainer (Leica Microsystems). For qRT-PCR analyses areas of ~1000 cells were microdissected from the arterial intima and media of ITA and RA from four of the six patients (patient number three to six). LMD could not be performed with samples of patient one and two due to a limited sample mass in these patients. Using a relatively constant number of cells for LMD, isolation of cells from each part yielded comparable quantities of RNA. Total RNA was extracted using the miRNeasy® FFPE kit (Qiagen, Hilden, Germany) and reverse-transcribed in a final volume of 20 µl using random hexamer primer (final concentration 25 ng/µl) and the SuperScript® II Reverse Transcription kit (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. As formalin-fixation is known to cause RNA fragmentation additional primer pairs which amplify shorter fragments were designed for each of the anti-apoptotic genes using Primer3 software (supplementary Table 2).

3.7 *Ex vivo* perfusion system

HSVGs of 61 patients were mounted in the perfusion device (Dummler *et al.*, 2011) and adjusted to a length matching the *in vivo* conditions. Total time from the operating room to the mounting of the vessels was less than one hour. The perfusion medium was DMEM/Ham's F-12 (PAA, Marburg, Germany) supplemented with 10% FCS, 2mM glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). In long-term experiments the medium was replaced every two days. The mounted veins were perfused under venous (flow: 5 ml/min, 10mmHg, n=26) or arterial conditions (flow: 50 ml/min, 100mmHg, n=24) for one to up to 14 days. At the end of each experiment, the fixed ends of the vein were discarded. The major part of the vessel was snap-frozen in liquid nitrogen and stored at -80°C until further use. The minor part of the vein was fixed in 4% PBS-buffered formalin (pH 7.4) for histology.

3.8 Zymography

MMP-2 protein activities were evaluated by a standard gelatine zymography. Briefly, 100 mg of frozen HSVG tissue were homogenized in ice cold zymography buffer (150 mM NaCl, 1 µm $ZnCl_2$, 1.5 mM NaN_3, 20 mM $CaCl_2$, 0.01% Triton X-100, 10 mM cacodylic acid, pH 5.0). Samples were centrifuged at 4°C for 10 min at 20.000 x *g*. The supernatant containing proteins was transferred to fresh Eppendorf tubes and stored at -80°C until further use. Ten µg of extracted protein were mixed with zymogram loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue) and separated in 15% SDS-PAGE gels containing 1 mg/ml type A gelatine from porcine skin (SIGMA-Aldrich, Taufkirchen, Germany). To renature proteins, gels were washed two times in 2.5% Triton X-100 for 15 min at room temperature and subsequently incubated in developing buffer, pH 7.5 (200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 0.02% Brij-35) overnight at 37°C. Gels were stained with 0.5% Coomassie Blue R250 in 40% methanol/10% acetic acid for 15 min and destained in 40% methanol/10% acetic acid until clear bands of lytic activity appeared. The reaction was stopped by transfer of gels in aqua bidest.

3.9 Statistical analysis

For the analysis of gene expression levels the comparison was made using the unpaired Student's t-test or using the relative expression software tool REST© (Pfaffl *et al.*, 2002). Differences in the vessel viability were calculated using the Mann-Whitney U-Test. Differences were considered to be significant at values of $p < 0.05$.

4. Cumulative thesis

Summary and individual contribution for achievement

This cumulative thesis consists of the following papers which were published during the PhD work:

4.1 Identification of an Up-regulated Anti-Apoptotic Network in the Internal Thoracic Artery (study I)

Previous studies comparing atherosclerotic and non-atherosclerotic arteries were limited by the fact that they compared vessels obtained from different patients. The specific aim of my first publication (Krane *et al.*, 2011) as equally contributing first author was, therefore, to identify molecular signatures or candidate genes which discriminate the atherosclerosis-resistant ITA from the atherosclerosis-prone RA, both derived from the same patient.

Firstly, I performed histomorphometric analyses with FFPE sections of ITA and RA from six different patients undergoing CABG surgery. I subsequently analyzed three commonly used morphometric parameters (ITI, IMR and %LN) revealing a significantly higher degree of intimal thickening in the RA compared to the ITA. Parts of the same vessels were used for RNA extraction and subsequent microarray analysis, which identified 552 genes differentially expressed between ITA and RA. The 418 up-regulated genes in the ITA showed annotations in several categories such as proliferation, cytokines and chemotaxis. The 35 genes with potential anti-apoptotic activity were annotated with high significance and an anti-apoptotic pathway generated from this cluster of genes was selected for further analysis. As an independent method I performed a qRT-PCR analysis which confirmed the significant up-regulation of the anti-apoptotic candidate genes MCL-1, p21, IER3, SOCS3, IL-6 and CCL2 in the ITA in all six patients. To address the question of the cellular origin of these genes I have subsequently used a laser microdissection approach on FFPE sections. My results show an up-regulation of all candidate genes in the intima of the atherosclerosis-resistant ITA suggesting that they may protect these vessels from the development of early signs of atherosclerosis, at least in part.

4.2 Pulsatile *ex vivo* perfusion of human saphenous vein grafts under controlled pressure conditions increases MMP-2 expression (study II)

Having identified several candidate genes with a potential anti-apoptotic activity in human arteries harvested during CAGB surgery, I tried to establish a system which is able to reflect the situation *in vivo* after the surgery as close as possible in order to evaluate the expression of these genes. Therefore, the second publication as equally contributed first author (Dummler *et al.*, 2011) is focused on the design and establishment of a novel *ex vivo* perfusion system which uses standardized and strictly controlled hemodynamic parameters for the pulsatile perfusion of HSVGs. After construction of the *ex vivo* perfusion system I used endoscopically harvested nonvaricose HSVGs from 35 patients undergoing CABG surgery to establish the perfusion device. At first, I tested cell viability of the perfused HSVGs with MTT staining and was able to show, that after perfusion with a physiological low-pressure profile (10 mmHg, 5 ml/min) the grafts maintained their viability for almost two weeks. In contrast, perfusion with arterial pressure conditions (100 mmHg, 50 ml/min) led to a dramatic reduction in viability after one week of perfusion. Furthermore, I could show on histological sections that arterial pressure profiles generated a visible thickening of the intima after three days and an extensive thickening after five days of perfusion.

I used the largest part of the mounted and perfused HSVGs for gene expression studies. Gene expression analyses of MMP-2 revealed a significant increase after exposure of HSVGs to arterial pressure profiles for three and five days of perfusion. We could show that this change in gene expression was also reflected on the protein level. Zymographic analyses revealed a similar strong and significant increase in gelatinolytic activity of MMP-2 after three and five days of arterial perfusion. Thus, the novel *ex vivo* perfusion system proved its ability to monitor alterations in the expression of genes which are expected to increase their activity due to elevated pressure conditions on the RNA and protein level. Therefore, our perfusion system is able to reflect the *in vivo* situation and monitor alterations in the activity of genes which are activated by arterial pressure and contribute to vascular remodeling. It might be a helpful tool to more precisely understand the molecular mechanisms associated with the arterialization of the vein and the development of IH leading to an early failure of HSVGs.

4.3 Additional Results

4.3.1 Additional Results: Arterial Bypass Grafts

To test the ubiquitous applicability and relevance of the anti-apoptotic network, I repeated the morphometric and mRNA gene expression analyses with eight ITAs and nine RAs from 14 different patients (clinical parameters can be found in Table 3). The analysis of the three morphometric parameters mentioned above to show the presence or absence of IH revealed significant differences between the two types of arteries and a stronger thickening in the RA compared to the ITA in all three parameters (Table 4) and are, therefore, in accordance with my previously results.

With this material I measured the expression of the previously analysed six genes from our anti-apoptotic network along with three additional genes (SOCS2, PROK2 and PIM1), and two other genes which are known to be linked to atherogenesis. On these independent samples the nine genes of the anti-apoptotic pathway showed similar results as before, but with an overall higher gene expression (Figure 5). Significant up-regulation was observed for SOCS2 (4.1-fold, p=0.02), IER3 (6.8-fold, p=0.01), MCL-1 (8.4-fold, p=0.002), IL-6 (9.5-fold, p=0.02), SOCS3 (10.5-fold, p=0.003), p21 (15.4-fold, p=0.004), PROK2 (17.6-fold, p=0.006), CCL2 (18.9-fold, p=0.001) and PIM1 (22.99-fold, p=0.004) in the ITA compared to the RA. Therefore, the dysregulated expression of these genes appears to be an event which generally takes place in vessels which are devoid of histological signs of atherosclerosis.

The pro-apoptotic gene p16 (CDKN2A) on the other hand showed a significant upregulation of gene expression in the RA compared to the ITA (3.8-fold, p=0.03). TNC, which was up-regulated in the RA in our microarray experiments, showed a likewise significant up-regulation in the RA compared to the ITA in the qRT-PCR analysis with a fold-change of 6.5 and a p-value of 0.01 (Figure 5).

Table 3: basic clinical characteristics of the ITAs and RAs from different patients selected for qRT-PCR analysis.

Patients	Age (years)	BW (kg)	Height (cm)	BMI	DM	HC	AH	FH CAD	EF (%)	CNA	No. BG
ITA 1	51	75	176	24.19	no	yes	yes	Yes	65	no	3
ITA 2	48	86	176	27.74	no	yes	yes	Yes	84	no	3
ITA 3	73	80	172	27.03	no	yes	yes	No	40	no	3
ITA 4	45	68	162	25.95	no	yes	yes	Yes	73	yes	4
ITA 5	78	68	175	22.22	no	no	yes	No	?	no	3
ITA 6	62	74	173	24.75	yes	no	yes	No	46	yes	4
ITA 7	65	85	178	26.83	yes	no	yes	No	62	yes	3
ITA 8	61	99	176	31.94	yes	no	yes	No	71	no	3
RA 1	68	88	186	25.14	no	yes	yes	No	62	no	3
RA 2	47	104	180	32.10	no	yes	yes	yes	58	no	3
RA 3	60	84	170	29.07	yes	yes	yes	no	75	no	3
RA 4	64	78	170	26.99	no	yes	yes	no	55	yes	2
RA 5	78	68	175	22.22	no	no	yes	no	?	no	3
RA 6	62	74	173	24.75	yes	no	yes	no	46	yes	4
RA 7	65	85	178	26.83	yes	no	yes	no	62	yes	3
RA 8	75	57	163	21.43	no	yes	yes	no	65	no	1
RA 9	55	82	180	25.31	no	yes	no	no	57	no	4

BW, Body weight; BMI, body mass index; DM, Diabetes mellitus; HC, Hypercholesterolemia; AH, Arterial Hypertension; FH CAD, Family history of coronary artery disease; EF, ejection fraction. CAN, Case history of nicotine abuse; No. BG, No. Of Bypass grafts; * ITA and RA from one patient

Table 4: Intimal thickening in the ITA and RA of different patients. Data represent mean value ± SD.

	ITA (n=8)	RA (n=9)	p-value
%LN	12.74 ± 2.76	33.88 ± 13.01	0.001
ITI	0.13 ± 0.06	0.26 ± 0.06	0.001
IMR	0.16 ± 0.06	0.63 ± 0.34	0.005

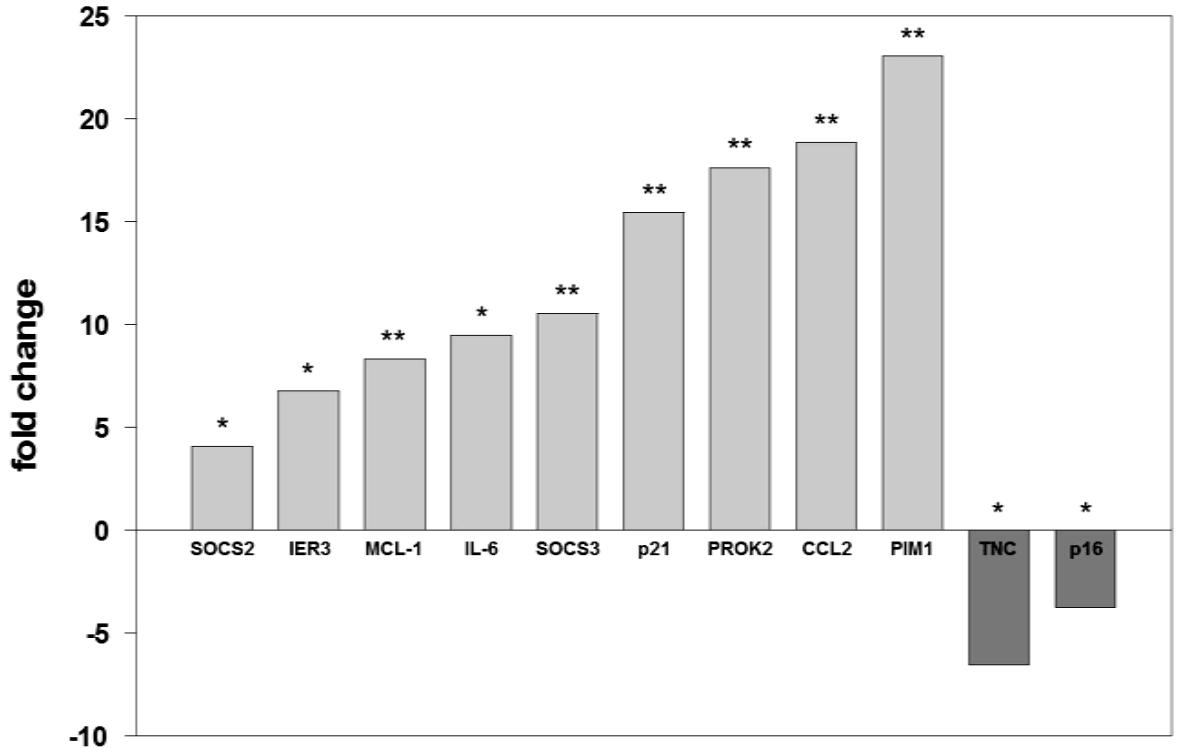

Figure 5: Anti-apoptotic genes showed an elevated expression in the atherosclerosis-resistant ITA. The downregulation in p16 and TNC demonstrate an overexpression in the atherosclerotic-prone RA. *p<0.05; **p<0.01

4.3.2 Additional Results: Venous Bypass Grafts

In addition to the published results (Dummler *et al.*, 2011), I was able to further demonstrate the development of NIH in HSVGs of 26 patients after perfusion under venous or arterial pressure conditions for one to five days by exploring potential changes in vessel wall integrity. No evidence of changes in wall integrity was found in the unperfused controls or the venously perfused vessels (data not shown). Exposure to venous pressure conditions for three days revealed no alterations and even after extension to five days only a minor thickening of the intimal layer was evident (Figure 6). Arterial perfusion for one day revealed no major changes in the graft wall. However, after three days of arterial perfusion the intimal layer started to visibly thicken and an extensive hyper-proliferative area could be detected after five days (Figure 7).

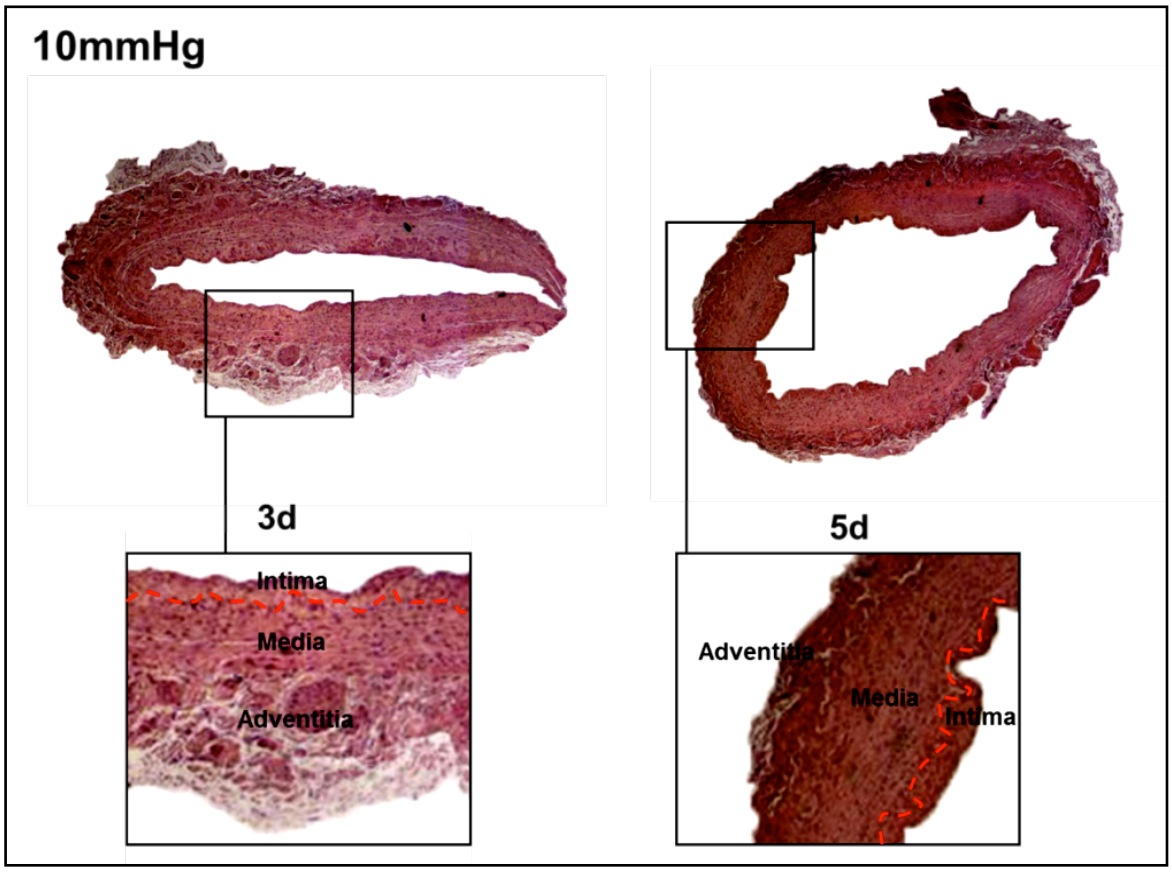

Figure 6: Hematoxylin and eosin staining of representative FFPE human SVGs after perfusion with venous pressure profile for 3 and 5 days showing no or only minor intimal thickening

The previously used three morphometric parameters ITI, IMR and %LN, which give evidence about the degree of intimal thickening, revealed significant differences between perfusion with 10mmHg or 100mmHg, respectively, (ITI p= 0.04, IMR p= 0.02, %LN p= 0.02) after five days. In contrast, after one and three days of perfusion, no significant differences could be observed between the two perfusion profiles in all three morphometric parameters (Table 5).

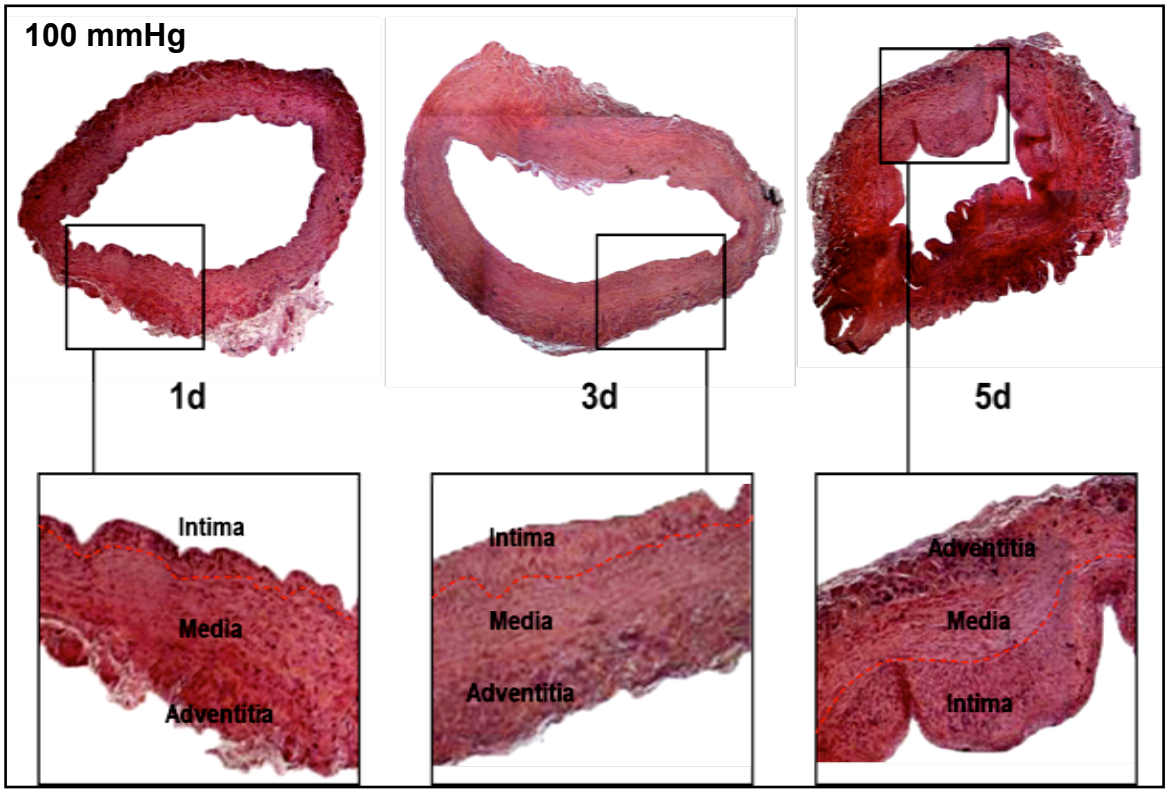

Figure 7: Hematoxylin and eosin staining of representative FFPE human SVGs after perfusion with arterial pressure profile for one, three and five days showing minor intimal thickening after three and strong thickening after five days of perfusion.

Table 5: Degree of intimal thickening in SVGs after perfusion under venous or arterial conditions for one to five days.

	ITI	IMR	%LN
10mmHg 1d	0.17±0.07	0.19±0.10	11.09±3.92
100mmHg 1d	0.19±0.07	0.22±0.12	13.48±0.75
10mmHg 3d	0.21±0.06	0.26±0.11	22.93±9.92
100mmHg 3d	0.21±0.05	0.15±0.05	22.95±3.21
10mmHg5d	**0.22±0.06**	**0.24±0.11**	**14.41±4.91**
100mmHg 5d	**0.54±0.29**	**0.71±0.36**	**40.84±20.05**

ITI = intimal area / medial area (intimal thickness index), IMR = maximal width of intima / maximal width of media (intima-to-media ratio), %LN = 100 x intimal area / lumen and intimal area (percentage of luminal narrowing).
Data represent mean values ± SD. Bold: p-value between arterial and venous perfused veins <0.05.

I also used FFPE sections from HSVGs after perfusion for 1, 3 or 5 days under venous or arterial pressure conditions for immunostaining with primary antibodies against CD31, Ki67 and caspase 3.

Immunostaining of the FFPE sections from HSVGs for the endothelial cell marker CD31 clearly demonstrated the presence of ECs and, therefore, intact intimal layers in negative control veins (Figure 8a) and HSVGs perfused for one day under venous and arterial pressure conditions (data not shown). The density of intact ECs, however, diminished over time with few ECs left after five days of perfusion under arterial pressure conditions (Figure 8a). Staining of FFPE sections of SVGs with antibodies against Ki67 did not reveal a nuclear staining in the control veins and after one day of perfusion. In contrast, nuclear Ki67 staining was observed mainly in the medial region after three days of perfusion under arterial perfusion conditions. After five days the staining became even stronger in the veins being perfused with arterial pressure conditions (Figure 8b).

Immunostaining for the apoptotic cell marker cleaved caspase 3 showed - similar to the results with Ki67 - a stronger staining and therefore a higher apoptotic rate in arterially perfused veins in contrast to the negative control or the venously perfused HSVGs. Immunostaining of cleaved caspase 3 was clearly visible after three days in the medial area of arterially perfused HSVGs. After five days of perfusion, staining was primarily concentrated to the media and to the boundary between the medial to intimal area (Figure 8c).

The pilot experiments performed with MMP-2 proved the *ex vivo* perfusion circuit to be a reliable system to detect alterations in mRNA expression. The results also suggested that perfusion for one day did not alter gene expression regardless of the pressure profile. Therefore, I investigated changes which were induced by arterial pressure profiles after five days and compared the expression to which was seen after one day of arterial perfusion. Based on the hypothesis that elevated pressure profiles would induce the expression of markers previously identified as anti-apoptotic candidate genes in the microarray analysis (Krane *et al*., 2011), I included several of these genes in qRt-PCR analysis. In addition, I speculated that the expression of pro-apoptotic genes might be diminished at the same time.

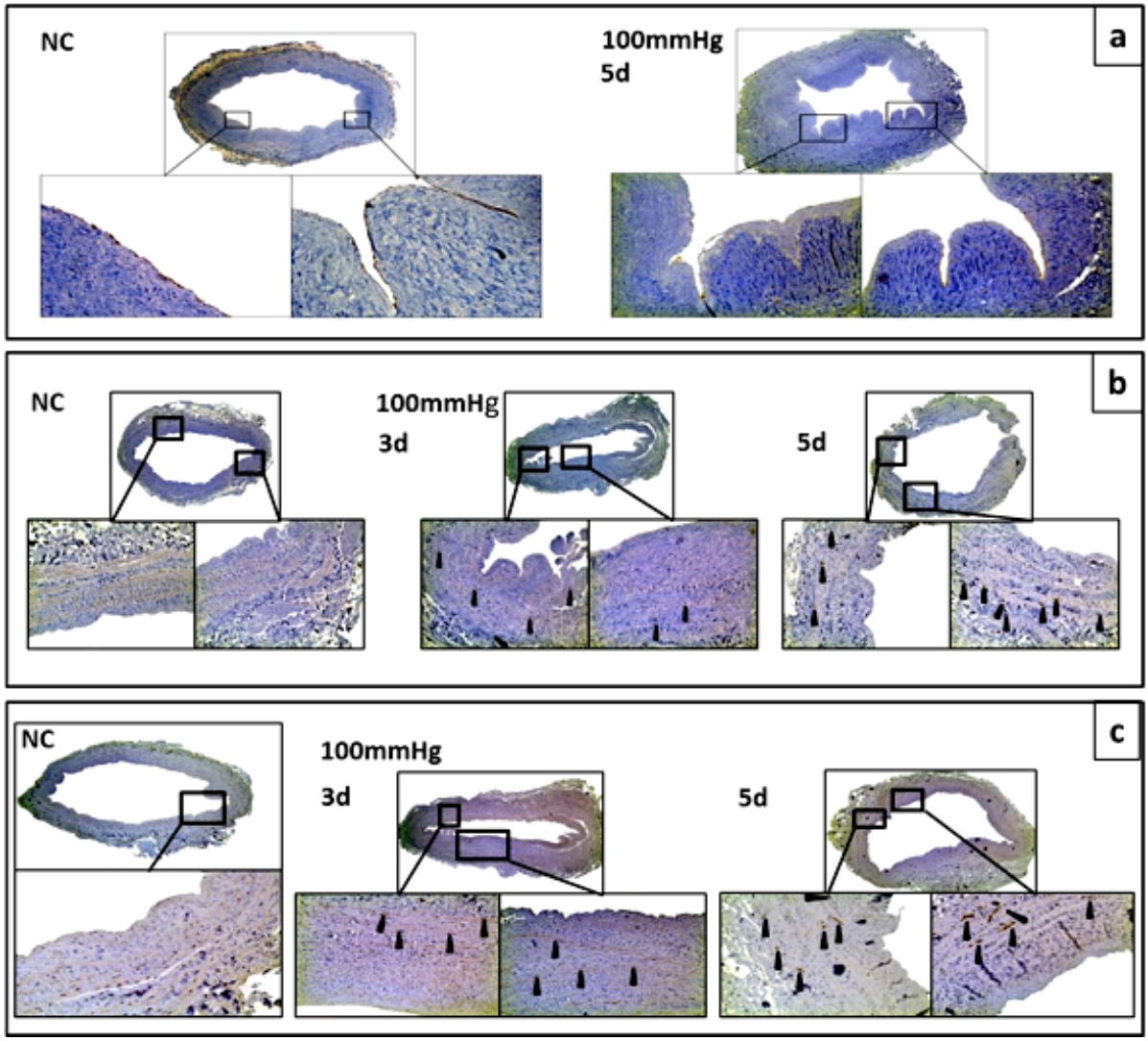

Figure 8: Characterization of SVGs submitted to an *ex vivo* pulsatile venous or arterial flow. Immunolabeling of human vein sections with antibodies against a: CD31, a specific marker of ECs demonstrated the presence of ECs along the vessel from freshly isolated veins (NC) to one day of perfusion under both pressure conditions, but only few intact ECs could be detected after 5d of arterial perfusion.; b: Ki67, a marker specifically expressed in proliferating cells revealed the presence of SMC proliferation in the neointima after three and five days of arterial perfusion; c: caspase 3, a marker specifically expressed in cells undergoing apoptosis revealed a higher apoptotic rate in the medial area of veins after arterial perfusion for five days. M = Media; L = lumen.

In fact four of five tested candidate genes (IL-6, MCL-1, SOCS3, p21) showed a strong up-regulation upon arterial perfusion. The most prominent elevation was seen with MCL-1 (more than 20-fold), followed by IL-6 (8-fold), SOCS3 (6-fold) and p21 (2.4-fold). Only the expression of IER3 with 0.9-fold remained rather unchanged (Figure 9). In contrast, the expression of two genes with supposed pro-apoptotic activity (BAX, p53) declined during extended perfusion with an arterial pressure profile with a fold-change of 0.4 for BAX and 0.2 for p53 (Figure 9). CCL2 showed

likewise a declined gene expression with a fold-change of 0.4. The EC marker VEGFA declined even further with a fold change of 0.01 and a reduced gene expression of more than 90% after five days of arterial perfusion (Figure 9).

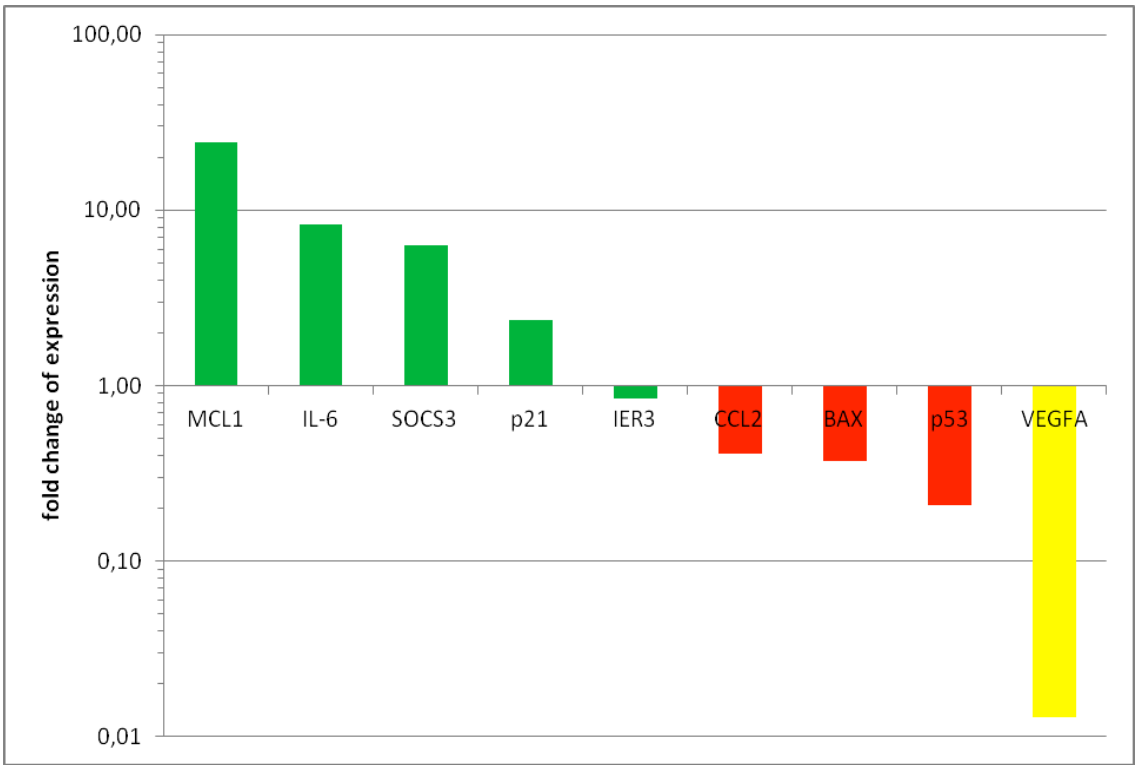

Figure 9: Gene expression ratio of: green, anti-apoptptoic; red, pro-apoptotic; yellow, EC function after perfusion of HSVGs under arterial (100mmHg, 50ml/min) conditions for five days compared to one day. The single five and one day values are relative to unperfused controls of the same veins, respectively. The gene/ß-actin ratio in unperfused veins was arbitrarily set to 1.

5. Discussion

The overall aim of my thesis was to identify differences in the gene expression profile of CABG bypass grafts which are related to the development of atherosclerosis and/or IH.

5.1 Arterial bypass grafts

In modern bypass surgery the atherosclerosis-resistant ITA is the first choice of bypass grafts since the 1980s (Lajos *et al.*, 2007; Lytle *et al.*, 1980) and the usage of bilateral ITAs is a standard procedure in CABG surgery.
Due to patency rates superior to those of human vein grafts other arterial bypass grafts like the RA are also taken for bypass surgery. With a patency rate of 89-94% (Passati *et al.*, 2003; Cohen *et al.*, 2001) atherosclerosis-prone RAs show patency rates inferior to that of the ITA (long term patency of 97-99). However, the patency rate is still clearly higher than in HSVGs (72-92%).
A main difference between the ITA and the RA is the presence of advanced intimal thickening and atherosclerosis in RAs at the time of surgery. Both arteries show a different morphology, which may be responsible for the increased vulnerability of RAs for IH. However, the underlying molecular mechanisms of this process are unclear at present and they were the major subject of these studies.

In a first approach, I compared mRNA expression levels in samples of the atherosclerosis-resistant ITA with samples of the atherosclerosis-prone RA from the same patient in order to identify genes possibly involved in atherogenesis or IH.
I used paired specimens of RA and ITA (n=6) which I first analyzed by histomorphometry to confirm a general difference in wall thickening between the two bypass grafts. The obtained morphometric data revealed a significant increase in the morphometric parameters IMR, ITI and %LN in all six RA compared to the ITA samples suggesting a higher grade of IH in the RA. My findings are in agreement with other studies showing a higher extent of IH and atherosclerotic plaque formation in the RA compared to the ITA (Kaufer *et al.*, 1997; Manabe & Sunamori, 2006;

Ruengsakulrach *et al.*, 1999; Malhotra *et al.*, 1996) with similar values for ITI and %LN. The IMR diverged slightly from our results possibly due to patients with an advanced atherosclerotic lesion type V, whereas our patients only showed lesion types II to III (Stary *et al.*, 1994 and 1995).

As our paired samples showed the expected histological differences, they were suitable to be used in a microarray analysis to detect atherosclerosis-related dysregulated genes.

The microarray analysis revealed 552 significantly differentially expressed genes between ITA and RA samples. Among the genes with a stronger expression in the ITA samples are various annotations enriched with high significance. The annotations were related to six main groups which partly overlap: development, extracellular matrix, signaling and receptors, inflammation and cytokines, chemotaxis and locomotion, cellular processes like proliferation and apoptosis (data not shown). A TreeRanker collection was made containing a more refined selection of the categories “apoptosis” (supplementary Table 3), “cytokines & chemokines”, “chemotaxis & locomotion”, and “cell proliferation” which were further studied in the pathway analysis using the PathwayArchitect™ software (Stratagene) to generate biological interaction networks (supplementary Figures 1 to 3 and Krane *et al.,* Fig. 1).

Apoptosis plays a constant and key role during the development of atherosclerosis from plaque formation, its progression and ultimately to plaque rupture (Mallat & Tedgui, 2000; Walsh *et al.*, 2000). Different cell types are involved in the apoptotic and anti-apoptotic mechanisms during atherogenesis. In particular, ECs in lesion-prone regions, where atherosclerotic plaques preferentially develop, are characterized by an increased apoptotic rate, leading to endothelial erosion and dysfunction (Kockx & Herman, 2000; Mallat & Tedgui, 2000; Durand *et al.*, 2004). Moreover, apoptosis of vascular SMCs is thought to promote plaque destabilization, as plaques tend to rupture at sites of reduced SMC content (Katsiki *et al.*, 2010). Apoptosis of SMCs in normal arteries is generally “silent”. However, when the atherosclerotic plaque has formed, apoptosis of SMCs renders the atherosclerotic plaques more vulnerable to rupture (Karaflou *et al.*, 2008).

Another important apoptotic process in atherosclerosis is macrophage death, which results in plaque rupture and acute vascular occlusion in advanced atherosclerotic lesions. In early lesions, macrophage foam cells undergo apoptosis and are then

rapidly scavenged by neighboring phagocytic macrophages. In late lesions, macrophages also undergo apoptosis and the post-apoptotic necrosis of the macrophages leads to increased inflammation, plaque instability and acute thrombosis (Karaflou *et al.*, 2008). Atherogenesis in the early phase is supported by a pro-apoptotic stimulation of ECs and an anti-apoptotic and proliferative stimulation of SMCs (Kutuk & Basaga, 2006). Throughout the whole process of atherosclerosis, both SMCs and ECs undergo programmed cell death (Tabas, 2007). Thus, experimental data strongly support the notion that apoptotic and anti-apoptotic processes represent a major mechanism responsible for regulation of the cellularity of the arterial wall during atherogenesis (Geng & Libby, 2002). The 35 genes with potential anti-apoptotic activity were annotated with high significance and the anti-apoptotic network comprising 11 genes (Krane *et al.*, Figure 1) generated from this cluster of genes was, therefore, selected for further analysis.

The results of the microarray analysis and the quantification by qRT-PCR both confirmed a significant up-regulation of MCL-1, p21, IER3, SOCS3, IL-6 and CCL2 in the athero-resistant ITA compared to the RA in all six patients. LMD revealed an elevated mRNA expression of our genes of interest in the intima of the ITA compared to the intima of the RA. Furthermore, comparing the relative expression levels between the intima and the media of the same artery, I could also show a consistently increased gene expression of our genes of interest in the intimal layer of both the ITA and the RA. Thus, these results strongly suggest that ECs in particular are the cellular source in which the pathway genes are highly expressed.

The up-regulated candidate genes of the pathway analysis have been shown to act in an anti-apoptotic manner. MCL-1 belongs to the B-cell lymphoma 2 (Bcl-2) family, which can be divided into two subgroups: the first group, composed of anti-apoptotic proteins (e.g. Bcl-2, Bclw and BclXL) and the second group which includes pro-apoptotic proteins (e.g. Bax, Bak, Bad), that antagonize the anti-apoptotic Bcl-2 family members. MCL-1 is known to block apoptosis and control the cell cycle through a mitochondria-related mechanism making the mitochondrial outer membrane susceptible for pro-apoptotic factors and it has been reported to delay cell cycle progression (Fujise *et al.*, 2000; Jamil *et al.*, 2005). Conditional knockout and promoter knock-in mouse models revealed that MCL-1 is essential for the survival of

many cell types in the organism including neutrophils, hepatocytes, hematopoietic stem cells or lymphocytes (Huang & Yang-Yen, 2010).

IER3 expression can be induced by many stimuli and is linked to various stress conditions like biomechanical strain of cardiomyocytes (Schulze *et al.*, 2003) or to the actions of cytokines, such as tumor necrosis factor alpha (TNFα) or IL-1b (Arlt & Schäfer, 2011). Despite these well-established mechanisms, the cellular effects of IER3 remain elusive, in particular the role of IER3 in apoptosis (Wu, 2003). The anti-apoptotic function of IER3 was first reported by Wu et al. in 1998, who reported an anti-apoptotic action by an unspliced variant of a gene termed IEX1L (Wu *et al.*, 1998) which is identical to IER3. IER3 was also found to be constitutively expressed in native non-injured vessels, but was barely detectable in intimal hyperplastic lesions induced by balloon injury and endothelial denudation (Lehoux & Tedgiu, 2003). Schulze et al. postulated that the induction of IER3 functions as part of a negative feedback mechanism limiting the local vascular response and the proliferation of VSMCs in mice subjected to carotid artery mechanical injury or in mice suffering from atherosclerosis and endothelial denudation due to LDL receptor deficiency. The vascular up-regulation of IER3 strongly inhibited neointima formation after vascular injury *in vivo* compared to controls (Schulze *et al.*, 2003). Being induced in the atherosclerotic or injured vasculature, the elevated expression of IER3 may contribute to the maintenance of vessel wall morphology and function after localised injury of the vessel wall (Arlt & Schäfer, 2011).

SOCS3 belongs to the family of suppressors of cytokine signalling (SOCS) proteins, which negatively regulate the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Alexander, 2002). SOCS proteins bind to janus kinase and to certain cytokine receptors and signalling molecules, thereby suppressing further signalling events like STAT3 phosphorylation. SOCS proteins play important regulatory roles in cardiovascular diseases, including intravascular coagulation, heart failure, and cardiovascular atherosclerosis. Studies have shown that SOCS proteins are key physiological regulators of inflammation (Tamiya *et al.*, 2011). Seki et al. (Seki *et al.*, 2000) found elevated levels of phosphorylated STAT3 in the medial and neointimal SMCs after balloon-injury in rat carotid arteries, whereas no STAT3 expression could be detected in the intact arteries. Local treatment with a JAK2-specific inhibitor resulted in the inhibition of STAT3 phosphorylation, neointimal SMC replication and the reduction of neointima formation in the injured arteries. In a

subsequent study, Shibata et al. (Shibata *et al.*, 2003) inhibited endogenous STAT3 activation which resulted in reduced proliferation of SMCs and a 40% reduction in the intima/media area ratio of the arteries. By contrast, deletion of the SOCS3 gene results in hyper-activation of STAT3 and induces apoptosis (Lu *et al.*, 2006), suggesting that SOCS3 is a critical regulator capable of converting from a pro-apoptotic into an anti-apoptotic messenger (Alexander, 2002).

The gene p21 is a potent cyclin dependent kinase inhibitor (CDKN1A) and was the first identified CDK inhibitor (Harper *et al.*, 1993). It is known to affect cell cycle progression and is likely to play a role in limiting arterial cell proliferation (Yang *et al.*, 1996). Several studies have described a reduction of neointima formation by the expression of p21 using adenoviral vectors after balloon injury (Yang *et al.*, 1996; Tanner *et al.*, 2000). Condorelli et al. showed that the expression of a mutated form of p21 with an increased biological activity protected against restenosis in $ApoE^{-/-}$ mice by reducing SMC proliferation and macrophage infiltration of the lesion after adenovirus-mediated gene transfer (Condorelli *et al.*, 2001). p21 is considered to be an anti-atherogenic molecule and, therefore, a potential therapeutic target that can be used to treat or prevent atherosclerosis and NIH.

The enhanced expression of IL-6 and CCL2 observed in the ITA compared to the RA appears to be surprising as both molecules are potent mediators of inflammation and attract monocytes during early atherosclerosis (Kumar *et al.*, 1997; Chen *et al.*, 2009; Loppnow *et al.*, 2008). However, both factors also can antagonize apoptosis by up-regulation of anti-apoptotic proteins such as cFLIP (cellular FLICE-like inhibitory protein), Bcl-2 or Bcl-X (Roca *et al.*, 2009) or through activation of p38 mitogen activated protein kinase (MAPK) and subsequent stimulation of protein kinase C gamma (PKCδ) activity (Lee *et al.*, 2009). Furthermore, the activity of CCL2 and subsequent production of IL-6 mediated survival of fibroblasts in lung tissue (Liu *et al.*, 2007). Thus, it is tempting to speculate that the combined up-regulation of both genes may lead to similar consequences in the atherosclerosis-resistant ITA.

The early phase of atherogenesis is defined by the endothelial dysfunction and inflammation with prominent lipid retention. This pro-inflammatory microenvironment predominantly provokes a proliferative response for SMCs, followed by intimal migration and neointima formation. In addition, EC apoptosis is apparently associated with the development of EC dysfunction and early atherosclerotic lesions show an extensive EC turn-over with dysfunctional endothelial cells. Thus,

atherogenesis in the early phase is supported by a pro-apoptotic stimulation of ECs and an anti-apoptotic and proliferative stimulation of SMCs (Kutuk & Basaga, 2006). My results support the hypothesis of an anti-apoptotic network which is differentially and increasingly expressed particularly in the intima of the ITA thereby protecting the vessel against EC cell death and finally neointimal hyperplasia and atherosclerosis.

I was able to further verify my published results with additional morphometric and qRT-PCR analysis of ITAs and RAs from independent patients.
Histological staining and the analysis of the three morphometric parameters could likewise demonstrate significant differences between the two types of arteries and a both clearly visible and stronger intimal thickening in the RA compared to the ITA and are, therefore, in accordance with my previously results.
Gene expression analysis revealed a significant up-regulation of nearly all genes of the anti-apoptotic pathway in the ITA. The previously examined six anti-apoptotic genes IL-6, MCL-1, IER3, p21, SOCS3 and CCL2 were higher expressed in the ITA compared to RA and even more as the ITA/RA-gene expression ratio from vessels of the same patients, thus, supporting out earlier results.
Furthermore, SOCS2, PROK2 and PIM1, which are also part of the identified anti-apoptotic pathway, have shown to be significantly up-regulated in the ITA compared to the RA. These results are, therefore, in accordance with the microarray analysis of samples from the same patients.
SOCS2 also belongs to the suppressor of cytokine signaling family and is known to be a regulator of somatic growth through regulation of growth hormone (GH)/insulin-like growth factor-1 (IGF-1) signaling. Several studies indicate that SOCS2 also has important actions in the central nervous system, the regulation of metabolism, the immune response, the mammary gland development, cancer, and other cytokine-dependent signaling pathways. (Rico-Bautista *et al.*, 2006). Consistent with the role of cytokines in human physiology, any SOCS2 imbalance could result in a broad range of pathologies such as cardiovascular diseases, insulin resistance, cancer, and severe infections. SOCS2, like the other members of the family, is able to regulate the cytokine-dependent JAK/STAT signaling pathway in several systems *in vitro* and has been associated with the regulation of genes like GH, IGF-1, IL-2 or IL-3, either positively or negatively. (Kato *et al.*, 2006). To date, there is no evidence of a direct involvement of SOCS2 in atherogenesis or IH, but it has been shown to affect

endothelial cell response to fetal plasma in placental vascular disease (Wang *et al.*, 2003).

PROK2 is one of the two discovered Prokineticin peptides, which are expressed in a wide variety of human tissues (Ngan & Tam, 2008). Prokineticins are involved in neuron migration and survival, angiogenesis, hematopoiesis and inflammation (Monnier & Samson, 2010). They act as survival factors for certain tissue-specific cells, and have the ability to induce angiogenesis and coordinate pro-inflammatory immune response (Monnier & Samson, 2010; Nebigil, 2009).

Environmental (hypoxia) and secreted factors (inflammation) as a response of tissue damage, activate the expression of prokineticins and their receptors and, therefore, link them to vascular remodeling. PROK2 is also a potent chemoattractant for monocytes and neutrophils and induces survival, differentiation and activation of the granulocytic and monocytic lineages (Monnier & Samson, 2010). Overexpression of PROK1 up-regulates PROK2, which in turn acts as a paracrine factor to activate progenitor cells to proliferate and differentiate into ECs and VSMCs (Nebigil, 2009).

The proto-oncogene PIM1 is known to be involved in multiple human cancers, cell cycle progression, apoptosis, survival and proliferation of hematopoietic cells and transcriptional activation. Its expression is induced by a variety of cytokines, growth factors, and mitogens suggesting that PIM1 may be an important intermediate messenger in signal transducing pathways such as JAK/STAT pathway (Bachmann & Möröy, 2005). It has also been shown that PIM1 activates p21 (Wang *et al.*, 2002) and that the anti-apoptotic factor Bcl-2 is up-regulated downstream of PIM1, suggesting that PIM1 is involved in cell cycle regulation and apoptosis (Rahman *et al.*, 2001). In addition, the requirement of PIM1 for differentiation of embryonic stem cells into ECs and VSMCs has recently been shown (Zippo *et al.*, 2004). Katakami et al. could observe PIM1-positive cells in the neointima of balloon-injured rat carotid arteries after immunohistochemical staining. They located PIM1 expression predominantly in the thickened intima of human thoracic aortas and coronary arteries. The specific inhibition of PIM1 function with a dominant negative PIM1-expressing adenovirus markedly suppressed neointima formation after balloon injury in vascular SMCs and their proliferation, suggesting a role for PIM1 in VSMC proliferation (Katakami *et al.*, 2004). Given its role in apoptosis, gene expression of PIM1 could possibly activate p21 in our case, thereby reducing SMC proliferation as we could not see intimal wall thickening in the ITA.

In addition to the nine genes of the anti-apoptotic pathway, I examined gene expression of p16 and TNC, which have shown to be up-regulated in the RA of samples from the same patients after microarray analysis.

The pro-apoptotic gene p16, which is up-regulated in the atherosclerosis-prone RA in our study, plays an important role in regulating the cell cycle. Mutations in p16 increase the risk of developing a variety of cancers, notably melanoma (Haluska & Hodi, 1998). The cyclin dependent kinase inhibitor is known to block cellular proliferation and governs the G_1/S cell cycle checkpoint, which is essential for determining whether a cell is arrested in G_1 or enters the S phase. Permanent cell cycle arrest or senescence has been associated with EC dysfunction and atherosclerosis. It has been shown in humans that blood vessels with atherosclerotic plaques contain a higher proportion of senescent vascular SMCs and ECs, as compared to non-atherosclerotic vessels (Douville *et al.*, 2011). It is observed that the expression of p16 increases with age and contributes to age-dependent senescence of some stem and progenitor cells (Yang *et al.*, 2008). Advanced age itself is an atherosclerotic risk factor, and is characterized by elevated oxidative stress and accumulation of ROS, which in turn may also increase the expression of phosphorylated p16 in endothelial progenitor cells (EPCs) (Yang *et al.*, 2008).

Tenascin C (TNC) is an extracellular matrix glycoprotein (Minear *et al.*, 2011) and has been shown to be up-regulated in the RA in both our microarray and qRT-PCR analyses. Little or no tenascin-C expression can be found in healthy adult tissue, but up-regulated expression was shown in sites of vascular disease (Golledge *et al.*, 2011; Midwood *et al.*, 2011). TNC expression has been linked to the development of IH, pulmonary artery hypertension, atherosclerosis, myocardial infarction, and heart failure (Golledge *et al.*, 2011; Minear *et al.*, 2011). TNC has been implicated in the development of IH following angioplasty, stenting, arteriotomy and bypass grafting in diverse animal species (Golledge *et al.*, 2011). It is expressed very rapidly following arterial injury in these models and its expression is reduced in situations where IH is inhibited. TNC expression was first described in atherosclerotic plaques by the group of Wallner et al. (Wallner *et al.*, 1999) and it appeared to rise with plaque progression. Microarray analysis confirmed the differential expression of TNC during atherosclerosis in human aortas with varying degrees of atherosclerotic levels (Seo *et al.*, 2004). Other studies showed similar results to our findings by comparing the human (Wallner *et al.*, 1999) or porcine atherosclerosis-resistant ITA (Qin *et al.*, 2007)

to coronary arteries, which are known to develop atherosclerosis. In addition to atherosclerosis, TNC expression has also been reported in the adventitia media of grafted human veins but not in normal veins (Midwood *et al.*, 2011). TNC expression seems to be transiently induced upon arterial injury, is continuously expressed during arterial diseases and even increases during atherosclerosis progression in plaque development correlating with inflammation and plaque rupture (Midwood *et al.*, 2011).

In conclusion, my experiments using independent ITAs and RAs from different patients confirm that the candidate genes identified by microarray analysis (IER-3, MCL-1, IL-6, SOCS3, p21, CCL2) appear to be commonly higher expressed in arterial vessels without atherosclerotic lesions or signs of IH. In addition, I could confirm the up-regulation in gene expression of SOCS2, PIM1 and PROK2 from our pathway which also may exert anti-apoptotic activity. These results are in line with the hypothesis that overexpression of a network of anti-apoptotic genes may protect these vessels from the development of early arteriosclerotic lesions. This is further supported by the fact that two genes with pro-apoptotic activity (p16, TNC) are down-regulated in the same samples.

5.2 Venous bypass grafts

Besides the ITA, HSVGs are still the common choice for CABG surgery and the treatment of peripheral artery diseases (PAD), because HSVGs are easy to use as a bypass graft due to their large diameter and wall characteristics (Sabik, 2011).
Nevertheless, up to 50% of the vein grafts fail during the first year of implantation. These first months are the most active period with respect to alterations in biological activity and wall remodeling (Owens, 2010). Although IH occurs in all implanted veins due to graft injury during surgical harvesting, only some locations of the grafts become severely stenosed while veins stay open in other regions (Owens, 2010). Surgical excision of the vein represents a vein injury model with altered endothelial functions which might directly impair the vein graft patency (Hinokiyama *et al.*, 2006).
The molecular mechanisms leading to the development of IH during the early stage of implantation in an arterial environment might give a first hint whether vein graft failure may occur.
The purpose of the second part of my work was, therefore, the design and establishment of a novel *ex vivo* perfusion system, which uses standardized and strictly controlled hemodynamic parameters for the pulsatile perfusion of HSVGs. With the *ex vivo* system, I was able to demonstrate the transfer of the *in vivo* situation after CABG surgery to the bench and evaluate whether hemodynamic forces may alter endothelial functions or provoke IH in a vein bypass graft. The culture system provides the ability to reproduce the initial events taking place when the grafted vein is exposed to arterial hemodynamic conditions and to identify the molecular markers which are involved in the development of IH during the process of “arterialization” of graft occlusion and failure within the arterial environment after CABG surgery.

The *ex vivo* perfusion system is suitable for long time trials by maintaining a constant mean pressure. Using MTT conversion I was able to confirm that HSVGs, which were perfused with a low-pressure profile in the system, remained viable for up to two weeks. This is in good agreement with other reports which have estimated the integrity of the vessels by histological or immunohistochemical methods (Miyakawa *et al.*, 2008; Paroz *et al.*, 2004; Rey *et al.*, 2004). Switching the conditions to an arterial pressure profile led to a visible reduction of the MTT staining beyond five

days of perfusion. These findings are similar to those of Miyakawa et al. who detected diminished cell viability in vein segments after perfusion with arterial conditions for four days (Miyakawa *et al.*, 2008). They confirmed their results by hematoxylin staining which also reveals a reduction of nuclear staining on day four. Gusic et al. could even show a dramatic increase in cell death index in all layers of the graft after one week (Gusic *et al.*, 2005). HE staining of the perfused HSVGs in our system showed a similar and clearly visible thickening of the intima after three days and an extensive thickening after five days of arterial perfusion.

In addition to the published results (Dummler *et al.*, 2011), I was able to further demonstrate the development of NIH in HSVGs of 26 patients after perfusion under venous or arterial pressure conditions for one to five days by exploring potential changes in vessel wall integrity.
Histological staining and analysis of the three morphometric parameters ITI, IMR and %LN revealed significant differences between perfusion with 10mmHg or 100mmHg, respectively. In contrast, after one and three days of perfusion, no significant differences could be observed between the two perfusion profiles in all three morphometric parameters.
The data resemble those when comparing the ratio of the morphometric parameters in the atherosclerosis-protected ITA and the atherosclerosis-prone RA in my previous studies and clearly demonstrate intimal wall thickening of the bypass grafts. Other studies show a significant increase of intimal and sub-intimal thickening compared to control tissue after extended perfusion of SVGs for 14 days under low pressure profiles, but without pulsatile flow settings (Rey *et al.*, 2004).
Low shear stress in particular is thought to be the key regulator for developing IH in the vein grafts after a certain time of perfusion, though this could not be observed in our setting within five days of perfusion. However, Saucy and colleagues reported intimal thickening and medial thinning after seven and 14 days of pulsatile perfusion of HSVGs under arterial hemodynamic conditions (Saucy *et al.*, 2010). In our setting arterial pressure profiles generated a visible thickening of the intima already after three days of perfusion. The injury of the endothelial layer due to harvesting and the change in hemodynamic conditions during the perfusion of the vein grafts may explain the significant development of IH (Saucy *et al.*, 2010), even after this short period.

The change in hemodynamic forces and the level of shear stress on the grafted HSVGs during arterial perfusion stimulates vessel wall proliferation and remodeling. With the formula for shear stress calculation by Gan et al. (Gan *et al.*, 1999)

$$\tau = \frac{1}{2} \times \left(\frac{\Delta P}{L}\right)^{\frac{3}{4}} \times \left(\frac{8 \times \eta \times Q}{\pi}\right)^{\frac{1}{4}}$$

where ΔP is the pressure which drops over the tube segment, η reflects the viscosity of the medium, Q stands for the flow rate and L represents the length of the vessel segment, I was able to calculate the mean wall shear stress in our perfused HSVGs. Mean wall shear stress for veins under venous perfusion conditions (5 ml/min, 10mmHg) revealed 0.32 dyne/cm^2. Viscosity of the cell culture medium is set with η=1.2 and the length of the perfused veins is considered to be of an average value of 2.0 ± 0.49 cm. For arterial perfusion of human SVGs (50 ml/min, 100mmHg) calculation of the mean wall shear stress yielded 11.4 dyne/cm^2. Our *ex vivo* perfusion system therefore reflects the *in vivo* situation of high wall shear stress on arterial and low wall shear stress on venous sites.

High shear stress stimulates thickening of the wall (Paszkowiak & Dardik, 2003). Long-term alterations in the maintenance and structure of vessel function by shear stress occur through regulation of protein synthesis and gene expression. Chronic exposure to pathological levels of shear stress may ultimately result in changes of the vessel wall that lead to atherosclerotic lesions or NIH. Some of these changes include the proliferation and migration of SMCs and expression of EC-surface molecules that stimulate leukocyte adhesion and migration.

I used these new histological sections to correlate the appearance of apoptosis and proliferative activity after venous and arterial perfusion at different time points.

Immunostaining of the FFPE sections from HSVGs for CD31 clearly demonstrated the presence of ECs and, therefore, intact intimal layers in negative control veins and HSVGs perfused for one day under venous and arterial pressure conditions. The density of intact ECs, however, diminished over time with few ECs left after five days of perfusion under arterial pressure conditions. Staining of FFPE sections of SVGs with antibodies against Ki67 did not reveal a nuclear staining in the control veins and after one day of perfusion. In contrast, nuclear Ki67 staining was observed mainly in the medial region after three days of perfusion under arterial perfusion conditions.

Together with the results of HE staining, these findings demonstrate major tissue proliferation with extensive thickening of the intimal layer mainly after five days of arterial perfusion. My findings are in accordance with those of Saucy and colleagues, who could also show a strong Ki67 staining and a high proliferation index in veins after perfusion with high pressure conditions for seven or even 14 days (Saucy *et al.*, 2010).

Immunostaining of cleaved caspase 3 as an apoptotic cell marker was clearly visible after three days in the medial area of arterially perfused HSVGs. After five days of perfusion, staining was primarily concentrated to the media and to the boundary between the medial to intimal area. *Ex vivo* perfusion of rat veins and subsequent immunostaining with cleaved caspase 3 antibody also demonstrated an apoptotic activity mainly in smooth muscle cells after perfusion with arterial pressure conditions in this model (Goldman *et al.*, 2003). Goldman et al. could demonstrate that caspase 3 was most notably activated by mechanical stretch in the *ex vivo* system assuming that the rapid, large mechanical stretch may mainly activate the p38-caspase 3 signaling pathway during the early stage of exposure to arterial blood pressure.

Overall, the endothelium trauma due to harvesting and the change in hemodynamic conditions during the perfusion seems to alter proliferation and the apoptotic rate of cells in the intimal and medial layer of HSVGs resulting in vein wall remodeling and eventually in provoking IH which may directly impair the vein graft patency (Saucy *et al.*, 2010; Goldman *et al.*, 2003).

Several *in vitro* and *in vivo* studies have demonstrated changes in graft morphology, viability, cellular density or gene expression under arterial conditions (Gusic *et al.*, 2005; Berceli *et al.*, 2004; Mavromatis *et al.*, 2000; Miyakawa *et al.*, 2008; Jiang *et al.*, 2009). Saucy et al. used an *ex vivo* vein support system to perfuse HSVGs under arterial conditions which mimicked the *in vivo* conditions of shear stress, flow rate and pressure during a period of 7 and 14 days. They found significant IH and a marked increase in PAI-1 expression in the human veins after 7 and 14 days of perfusion (Saucy *et al.*, 2010). The group of Porter et al. demonstrated that arterial shear stress inhibited the development of IH in cultured vein pieces (Porter *et al.*, 1996).

Previous studies have shown that SMC proliferation and migration depend on the activity of matrix-degrading enzymes. In fact, MMP-2 is an enzyme which is directly

involved in vascular remodeling (Whatling *et al.*, 2004) and rodent animal models confirm that MMP-2 levels are increased under hypertensive conditions (Castro *et al.*, 2008; Derosa *et al.*, 2004).
Within three days of perfusion under arterial pressure conditions in our perfusion system the expression of MMP-2 increased more than nine-fold and reached an even higher value after five days, similar to the activation of PAI-1 (Paroz *et al.*, 2004).
My data are further supported by other reports showing an increased *de novo* synthesis of MMP-2 in HSVGs perfused under arterial conditions (Mavromatis *et al.*, 2000) or in animal models who underwent vein grafting (Berceli *et al.*, 2004; Thomas & Newby, 2010). The zymographic analyses are in accordance with their results and those of Patterson et al. (Patterson *et al.*, 2001). They suggest similar strongly increased gelatinolytic activities in veins after perfusion with arterial pressure profiles which is predominantly due to the induction of the active form of MMP-2.

In addition to MMP-2 and the second publication (Dummler *et al.*, 2011), I investigated changes in gene expression levels with other HSVGs after perfusion under different pressure profiles in order to identify molecular markers, which are involved in the development of NIH beyond normal “arterialization” patterns of the vein graft to gain a better understanding in the mechanisms finally resulting in vein graft occlusion.
The pilot experiments performed with MMP-2 proved the *ex vivo* perfusion circuit to be a reliable system to detect alterations in mRNA expression. The results also suggested that perfusion for one day did not alter gene expression regardless of the pressure profile. Therefore, I investigated changes which were induced by arterial pressure profiles after five days and compared the expression to which was seen after one day of arterial perfusion. It is well known that the initial molecular events after grafting the vein into an arterial hemodynamic environment with an acute increase in flow rates and intraluminal pressure take place rather rapidly within a couple of days. It appeared suitable to investigate alterations in the expression and the biological activity of molecules which are related to NIH, apoptosis and inflammation. Based on the hypothesis that elevated pressure profiles would induce the expression of markers previously identified as anti-apoptotic candidate genes in the microarray analysis (Krane *et al.,* 2011), I included several of these and other target genes in my analysis. Indeed a strong up-regulation of genes involved in the

development of IH and vein graft wall remodeling (MMP-2, CCL2) as well as pro-apoptotic genes (BAX, p53) could be recorded already after one day of perfusion, demonstrating a fast response to the altered pressure conditions and suggesting endothelial injury. Several of the anti-apoptotic genes showed a delayed response to the arterial pressure but a strong up-regulation could be demonstrated after five days. They might activate inflammation (IL-6, CCL2), inhibit apoptosis of SMCs and enhance their migration and proliferation into the intima (SOCS3, MCL-1, IER3) thereby promoting excessive IH. The pleiotropic cytokine IL-6 is induced early after vessel trauma by the mechanical stress of the surgery or by grafting it into an unphysiological environment with high shear stress. The BCL-2 related gene MCL-1 protects from apoptosis by binding the pro-apoptotic factor BAX. During perfusion for five days, MCL-1 expression gradually increased while expression of BAX decreased conversely. Therefore, the anti-apoptotic effect of MCL-1 may protect SMCs from apoptosis and allow their migration into the intimal area and the promotion of NIH. At the same time, the pro-apoptotic genes induce apoptosis of intimal cells early after vein graft transplantation. The pleiotropic factor CCL2 which was shown to act anti-apoptotic in arterial bypass grafts in a previous study (Krane *et al.*, 2011) showed a declined gene expression after perfusion in human venous grafts under arterial pressure conditions. In the literature, CCL2 is often described as endothelial adherence molecule, attracting leukocytes to the site of inflammation and vein graft adaption (Muto *et al.*, 2010; Schepers *et al.*, 2006). CCL2 also stimulates the migration and proliferation of quiescent SMCs into the vessel wall (Selzmann *et al.*, 2002; Zhu *et al.*, 2001) and Tatewaki et al. could demonstrate that CCL2 plays a central role in neointimal formation in vein graft failure after mechanical injury and in the development, progression and destabilization of atherosclerotic lesions (Tatewaki *et al.*, 2007).

The results with the *ex vivo* perfusion system suggest that perfusion with an arterial pressure profile for five days leads to an up-regulation of genes with potential anti-apoptotic activity while pro-apoptotic genes may be down-regulated. This would create an equilibrium shift of apoptosis-regulating factors to a point that the tissue might be protected from programed cell death but rather is committed to proliferation. This is in line with the hyperplasia seen in these vessels at this time. Finally, I have determined the expression of VEGFA as a marker to assess EC function. Immunohistochemical staining with anti-CD31 antibodies revealed a disruption of the

integrity of the EC layer after five days of arterial perfusion. Therefore, endothelial function should be reduced and the results obtained with VEGFA expression strongly support this notion. After five days VEGFA expression is reduced by more than 90%. Thus, the alterations obtained by the *ex vivo* perfusion system are in line with my previous results of the comparative microarray analysis between arteriosclerosis-prone and –resistant vessels (Krane *et al.*, 2011) and they are in good agreement with the results of the immunohistochemical evaluation.

With the *ex vivo* perfusion system I was able to show the up-regulation of several biomarkers in HSVGs after perfusion in an arterial environment which are linked to the development of IH in vein grafts *in vivo* and *in vitro*. MMP-2 is involved in the proliferation and migration of SMCs and therefore in vascular remodeling and the development of IH. The previously identified anti-apoptotic genes (Krane *et al.,* 2011) seem to activate inflammatory processes (IL-6, CCL2), inhibit apoptosis of SMCs and enhance their migration and proliferation into the intima (SOCS3, MCL-1, IER3) thereby promoting excessive IH. To prevent HSVGs from occlusion and failure after CABG bypass grafting, the examined genes could be potential therapeutical targets to reduce the inflammatory events taking place after grafting and inhibit SMC migration and proliferation to reduce or silence IH after the process of “arterialization”.

6. Summary and Conclusion

The focus of my thesis was the investigation and identification of molecular alterations which might potentially be involved in the development of atherosclerosis and/or IH in human CABG bypass grafts. The approach included a comparative genome-wide expression analysis of human arteries derived from the same patient with a complementary stage of atherosclerosis. My results have identified a consistent up-regulation of a panel of anti-apoptotic candidate genes in the intimal layer of the atherosclerosis-resistant internal thoracic artery. These data could be confirmed with independent ITA and RA samples from different patients. The activity of these genes may contribute to the protection of the ITA against the development of intimal wall thickening which in contrast could be seen extensively in the RA.

Furthermore, an *ex vivo* perfusion system using human saphenous veins was established which allowed the stable and well controlled perfusion with defined pressure profiles and subsequently the reliable measurement of molecular parameters during the very early stage of atherosclerosis. It appeared suitable to investigate alterations of gene expression and the biological activity of molecules which are related to NIH, apoptosis and inflammation. Therefore, this system was able to reflect the *in vivo* situation after CABG surgery from the bedside to the bench by placing the vessels into an environment which resembles the conditions *in situ* in the patient. It might be a helpful system to understand the underlying molecular mechanisms of early atherosclerosis thereby potentially improving the long-term patency rate of human bypass grafts.

Overall, my data show a similar up-regulation of anti-apoptotic candidate genes both in the atherosclerosis-protected ITA and in arterially perfused HSVGs. Despite this commonness, the activity of these genes can nevertheless differ completely in both vessel types.
In the ITA, the identified anti-apoptotic genes seem to block apoptosis in ECs, limit vascular response and SMC proliferation and, therefore, inhibit the development of early signs of atherosclerosis.

In human saphenous veins the candidate genes appear to act in an anti-apoptotic way as well. In these vessels, they may block apoptosis in SMCs rather than in ECs resulting in elevated proliferation and migration and finally an enhanced intimal thickening of the vessel walls.

The results of my work will help to better understand the molecular mechanisms and signaling pathways involved in the development of early atherosclerotic signs and NIH in bypass grafts with unsatisfactory patency rates (RA, HSVGs). The better understanding of these processes, the further study and unraveling of the athero-protecting mechanisms in the ITA may contribute to improve the clinical outcome after CABG surgery.

The *ex vivo* system might be well suited to gain more precise insight into the molecular mechanisms associated with the process of “arterialization” in the vein as a necessary response to adapt to the arterial pressure environment after surgery and the development of IH leading to an early failure of HSVGs.

New strategies to prolong the lifespan of vein grafts are definitely needed. This can only be achieved by a better understanding of the cellular and molecular events taking place during IH development. Future studies with our *ex vivo* system will be necessary to identify new candidate genes, which might be targets for novel therapeutic approaches. Perfusion of human arterial and venous bypass grafts with autologous mononuclear cells will help to identify the level of inflammatory cell attachment to the endothelium of vascular grafts and the expression of pro- and anti-inflammatory markers in the arterially perfused HSVGs. The perfusion of HSVGs with autologous human plasma will be an additional approach to overcome existing limitations allowing the examination of the inflammatory processes after grafting of the veins in an arterial pressure environment very close to the physiological conditions *in vivo*. The improvement of the perfusion system is a constant goal in order to optimize the perfusion and to extend the life-span of HSVGs under controlled conditions *ex vivo*.

7. Acknowledgements

The completion of this thesis has been a long journey and could have not been accomplished without the generous help, encouragement and support of numerous people including my, dear colleagues, family and friends. Here is a small tribute to all those people.

First of all, I would like to thank my supervisor Prof. Dr. Hans Hauner for accepting me as his Ph.D student and for his continuous support of my Ph.D study and research throughout the years. I am deeply grateful for his detailed and constructive comments, his patience and motivation.

I want to express my deepest gratitude towards my lab supervisor PD Dr. Harald Lahm, without his support and guidance this thesis would not have been possible. He has been actively interested in my work and has always been available to advise me. I am very grateful for his patience, motivation, enthusiasm, immense knowledge in molecular biology and his excellent writing skills (which helped to improve mine a lot) All this makes him a great mentor.

I owe my most sincere gratitude to Dr. med. Markus Krane, who gave me the opportunity to work in the Department of Experimental Surgery of the German Heart Center Munich. I thank him for his continuous support, his constructive criticism and his “positive way of thinking” during the preparation of this thesis, which helped me a lot on my way to get, where I am now.

I would like to thank my former lab supervisor Dr. Joachim Grammer, for his support, advice and encouragement, although our time together was rather short.

I would like to thank Dr. Steffi Doppler for being my friend and fellow traveler for so many years. For her professional and personal advice, her emotional support, her understanding, her encouragement, for being a friend for all those years.

Thanks to the present and past members of our lab: Christian Tesche who always took care of the late-night and weekend cell culture experiments without muttering. Thanks for that and of course for all the exceptional carnival parties! Martina Dreßen for giving me an introduction into the lab, her support, particularly in the early days, our discussions which gladly have not been work-related all the time and being my “roommate”, Wiesn’-fellow and friend for all those years. Stefan Eichhorn for his valuable contributions in constructing everything we came up with and really believing in our ideas, for sharing the same passion for our work and

for supplying me with the best honey, ever. Mrs. Angelika Bernhard for sharing her knowledge and helping me with my experiments. And I'd like to thank all the other members of the lab for creating a friendly working environment.

A part of this work was done in collaboration with Prof. Dirk Haller of the Biofunctionality Unit at the Research Center for Nutrition and Food Sciences in Weihenstephan. Thanks to my collaborators Dr. Michael Hofman and Katharina Heller for our joint effort to make the LMD work and for helping me with the Bioanalyzer.

I would like to express my sincere thanks to Prof. Dr. Rüdiger Lange and all the surgeons in the German Heart Center for providing me with sample material from human bypass grafts. And thanks to all the nurses, who made it possible that the samples reached us almost immediately.

I would like to thank all the people in charge at the TUM Graduate School for their financial and educational support, which enabled me to highly improve my soft skills.

For the non-scientific side of my thesis, I owe my loving thanks to my family.
My mother Xaveria Dummler, who inspired me from the beginning, who was and is my role model in so many things and who supported me all my life both, financially and emotionally. Without her, I would not be where I am, for sure.
My father Josef Dummler who always kept faith in me, unfortunately died much too young for both of us and whom I miss terribly. This work is also dedicated to you, I know, you would be exceedingly proud.
I want to thank my loving brother and sisters Baptist, Mayumi, Agnes and Viktoria, who lived through my emotional rapids, I had to move through and my nieces Emma and Romy for being my sunshine.

I particularly want to thank my partner Jochen Friedl for encouraging and loving me the last 8,5 years. The greatest support he could ever give me, was during the final step of my thesis, when he made the past 10 nerve-wrecking months during the writing process the best time of my life. I surely will return you the favor! I would also like to thank him (and his parents Karl and Inge) for the financial support.

Last but not least I want to thank all the people who have appeared in my life the last years and all the friends, that stayed. Thank you for all your support, the talking, the laughing, the memories… guys, we made it- the mission is completed. I THANK YOU ALL!"

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9. Appendix

9.1 Supplementary Figures

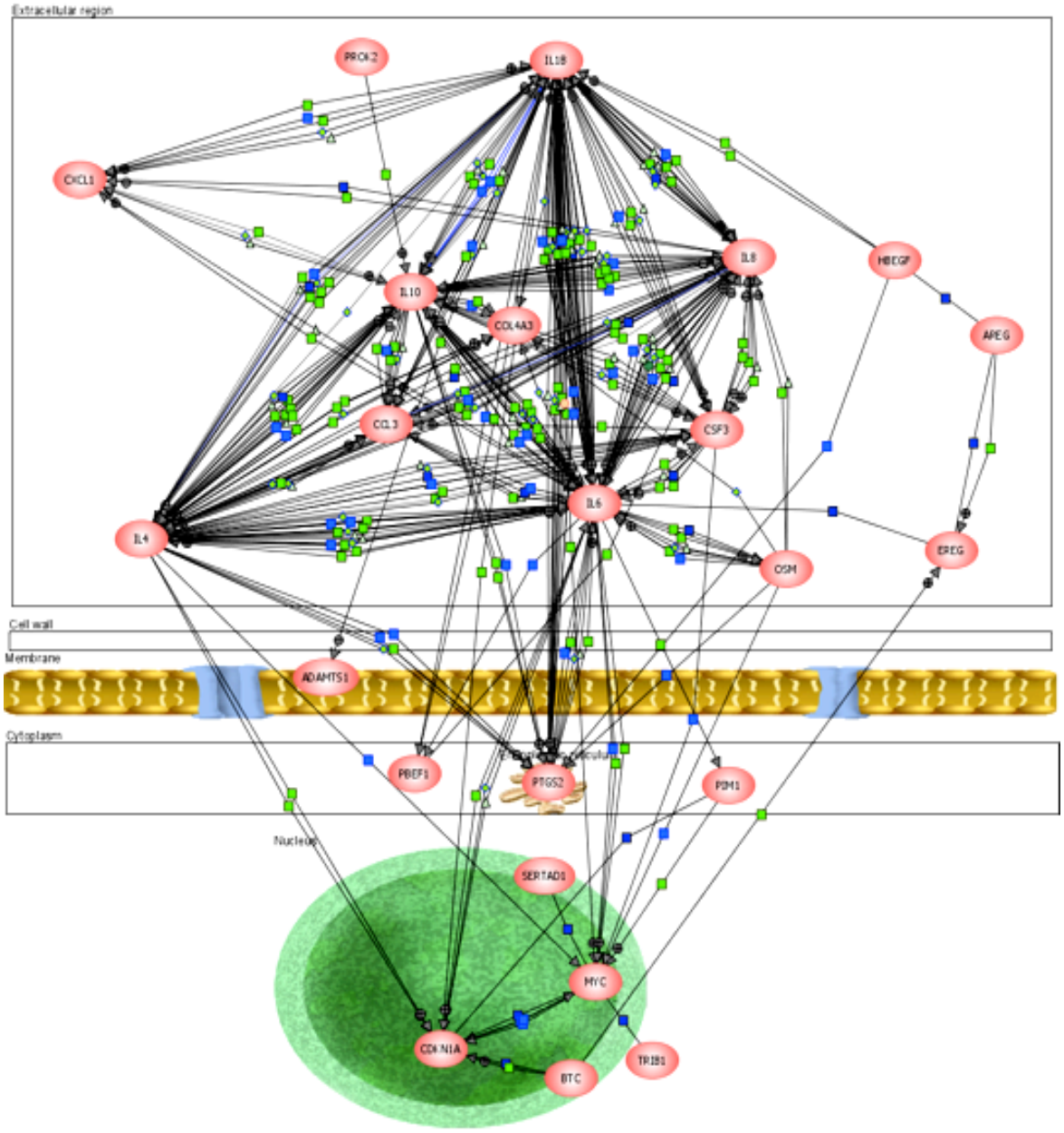

Figure 1: Biological interaction network of the genes related to cell proliferation as suggested by the PathwayArchitect software.

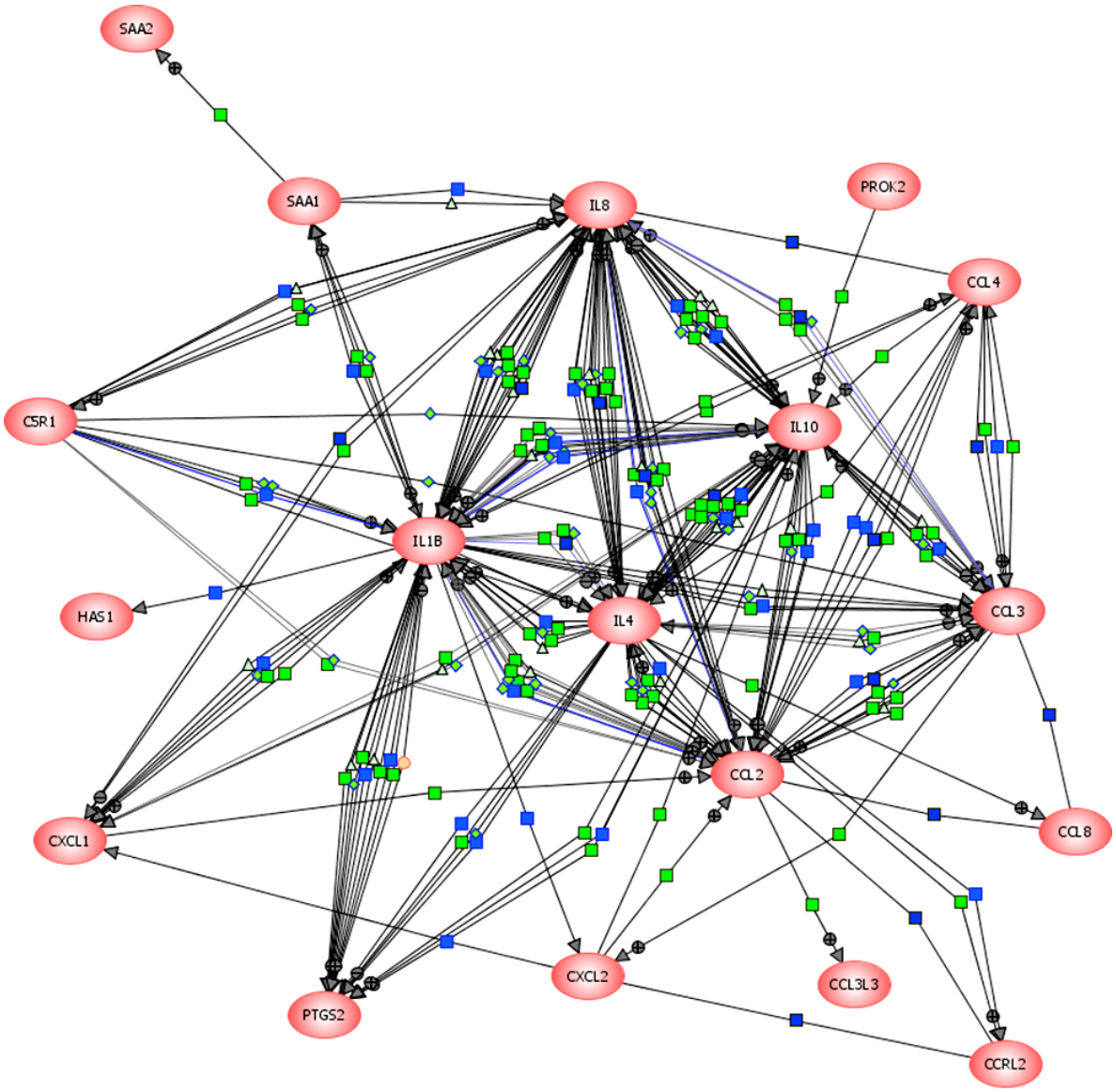

Figure 2: Biological interaction network of the genes related to chemotaxis as suggested by the PathwayArchitect software.

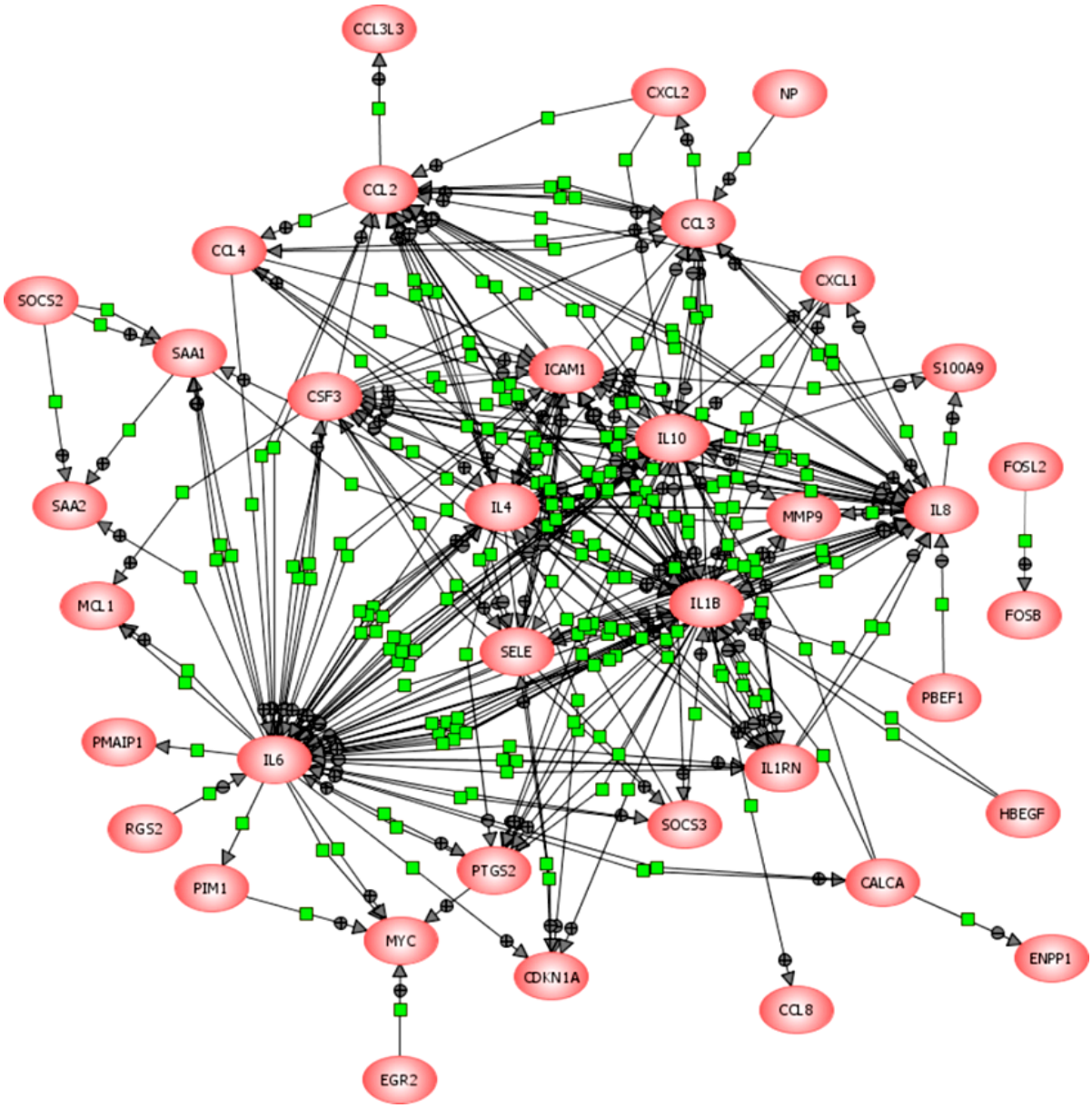

Figure 3: Biological interaction network of the genes related to (anti-)inflammatory cytokines as suggested by the PathwayArchitect software.

9.2 Supplementary Tables

Table 1: Immunohistochemistry methods

Immunostaining	**primary antibody**	**secondary antibody**	**Detection system**
Caspase 3	Cleaved Caspase 3 1:100	Biotynilated secondary antibody (Vectastain ABC-Kit)	Vectastain ABC-Kit Elite Universal (Biozol, Eching, Germany)
treatment	Paraffin removal Pre-treatment with Pro Taqs II Antigen-Enhancer Peroxidase blocking Blocking serum (Vectastain ABC-Kit) primary AB caspase 3 cleaved 1:100 Avidin Biotynilated enzyme complex (ABC) Enzyme substrate chromogen AEC (Invitrogen, Darmstadt, Germany) Counterstain with Hematoxylin & Eosin		
Ki67	Anti-human Ki67 MIB-1	EnVision™ FLEX /HRP detection reagent (Dako) Goat-anti-Mouse IgG	EnVision™ FLEX, High pH (Dako,Hamburg, Germany)
treatment	Paraffin removal Pre-treatment heat-induced epitope retrieval (HIER) with EnVision™ FLEX Target Retrieval Solution Peroxidase-Blocking primary AB mouse monoclonal anti-human Ki67 1:100 Dako EnVision™ FLEX /HRP detection reagent Enzyme substrate EnVision™ FLEX DAB+ Chromogen Counterstain with Hematoxylin & Eosin		
CD31	Anti-human CD31 Clone JC70A	EnVision™ FLEX /HRP detection reagent (Dako) Goat-anti-Mouse IgG	EnVision™ FLEX, High pH (Dako,Hamburg, Germany)
treatment	Paraffin removal Pre-treatment heat-induced epitope retrieval (HIER) with EnVision™ FLEX Target Retrieval Solution Peroxidase-Blocking primary AB mouse monoclonal anti-human CD31 1:30 Dako EnVision™ FLEX /HRP detection reagent Enzyme substrate EnVision™ FLEX DAB+ Chromogen Counterstain with Hematoxylin & Eosin		

Treatments: between each step: 3x 5min washing with 1% PBS or 2x 5min in TRIS buffer (Caspase 3)

Table 2: Primer pairs used for qRT-PCR (quantitative real-time polymerase chain reaction)

Gene	GenBank accession number		Primer sequences (5´-3´)	PCR product (bp)
Validation of MA-results				
p21	NM_000389	sense	5`-ggaagaccatgtggacctgt-3`	178
		anti-sense	5`-ggattagggcttcctcttgg-3`	
SOCS3	NM_003955	sense	5`-cccccagaagagcctattaca-3`	112
		anti-sense	5`-acggtcttccgacagagatg-3`	
IER3	NM_003897	sense	5`-cggtcctgagatcttcacct-3`	185
		anti-sense	5`-tggtgagcagcagaaagaga-3`	
MCL-1	NM_021960	sense	5`-taaggacaaaacgggactgg-3`	137
		anti-sense	5`-accagctcctactccagcaa-3`	
IL-6	NM_000600	sense	5`-agtgaggaacaagccagagc-3`	189
		anti-sense	5`-aaagctgcgcagaatgagat-3`	
IL-10	NM_000572	sense	5`-agctgtggccagcttgttat-3`	164
		anti-sense	5`-gtagagacggggtttcacca-3`	
CCL2	NM_002982	sense	5`-ccccagtcacctgctgttat-3`	135
		anti-sense	5`-agatctccttggccacaatg-3`	
GAPDH	NM_002046	sense	5`-gagtcaacggatttggtcgt-3`	185
		anti-sense	5`-gacaagcttcccgttctcag-3`	
Laser microdissection				
p21	NM_000389	sense	5`-cgaagtcagttccttgtggag-3`	110
		anti-sense	5`-catgggttctgacggacat -3`	
SOCS3	NM_003955	sense	5`-cccccagaagagcctattaca-3`	112
		anti-sense	5`-acggtcttccgacagagatg-3`	
IER3	NM_003897	sense	5`-cagccgcagggttctcta-3`	75
		anti-sense	5`-agccttttggctgggttc-3`	
MCL-1	NM_021960	sense	5`-caaaacgggactggctagtta -3`	54
		anti-sense	5`-agaactccacaaacccatcc -3`	
IL-6	NM_000600	sense	5`-gaagctctatctcgcctcca-3`	102
		anti-sense	5`-agcaggcaacaccaggag-3`	
CCL2	NM_002982	sense	5`-ccccagtcacctgctgttat-3`	135
		anti-sense	5`-agatctccttggccacaatg-3`	
β-actin	NM_001101	sense	5`-ccaaccgcgagaagatga-3`	96
		anti-sense	5`-ccagaggcgtacagggatag -3`	
Validation in ITA and RA samples of different patients				
SOCS2	NM_003877	sense	5`-cagtcaccaagccccttc-3`	108
		anti-sense	5`-aagggatggggctctttct-3`	
PROK2	NM_021935	sense	5`-tgccatccactgactcgtaa-3`	66
		anti-sense	5`-cagacatgggcaagtgtga-3`	
PIM1	NM_002648	sense	5`-atcaggggccaggttttct-3`	73
		anti-sense	5`-gggccaagcaccatctaat-3`	
p16	NM_058197	sense	5`-gtggacctggctgaggag-3`	132
(CDKN2A)		anti-sense	5`-ctttcaatcggggatgtctg-3`	
TNC	NM_002160	sense	5`-ccttgctgtagaggtcgtca-3`	66
		anti-sense	5`-ccaacctcagacacggcta-3`	
Ex vivo perfusion studies				
MMP-2	NM_004530	sense	5`-tgctggagacaaattctgga -3	90
		anti-sense	5`-gatggcattccaggcatc-3	
IL-6	NM_000600	sense	5`-gaagctctatctcgcctcca -3`	102
		anti-sense	5`-agcaggcaacaccaggag-3`	
MCL-1	NM_021960	sense	5`-aagccaatgggcaggtct-3`	121
		anti-sense	5`-tgtccagtttccgaagcat-3`	
SOCS3	NM_003955	sense	5`-cccccagaagagcctattaca-3`	112
		anti-sense	5`-acggtcttccgacagagatg-3`	
IER3	NM_003897	sense	5`-cggtcctgagatcttcacct-3`	185
		anti-sense	5`-tggtgagcagcagaaagaga-3`	

CCL2	NM_002982	sense	5`-ccccagtcacctgctgttat-3`	135
		anti-sense	5`-agatctccttggccacaatg-3`	
p21	NM_000389	sense	5`-cgaagtcagttccttgtggag-3`	111
		anti-sense	5`-catgggttctgacggacat-3`	
BAX	NM_004324	sense	5`-atgttttctgacggcaacttc-3`	104
		anti-sense	5`-atcagttccggcaccttg-3`	
p53	NM_000546	sense	5`-ttctgtcccttcccagaaaa -3`	125
		anti-sense	5`-gttggcaaaacatcttgttgag-3`	
VEGFA	NM_001025366	sense	5`-tcttcaagccatcctgtgtg-3`	123
		anti-sense	5`- ggtgaggtttgatccgcata-3`	
β-actin	NM_001101	sense	5`-ccaaccgcgagaagatga-3`	96
		anti-sense	5`-ccagaggcgtacagggatag-3`	

Table 3: Significantly enriched annotations related to “apoptosis/cell death” in the list of genes down-regulated in the comparison of RA and ITA samples (i.e. stronger expression in the ITA samples). “GO”: Gene Ontology; “GM”: GeneMapp

Name	Gene	GO:0016265 death	GO:0008219 cell death	GO:0006915 apoptosis	GO:0012501 programmed cell death	GO:0043066 negative regulation of apoptosis	GO:0043069 negative regulation of programmed cell death	GO:0042981 regulation of apoptosis	GO:0043067 regulation of programmed cell death	GM:hs_negative_regulation_of_apoptosis	GM:hs_negative_regulation_of_programmed_cell_death	Description	Annotation
ACTN2	A_23_P115021	X	X	X	X			X	X				actinin, alpha 2 (ACTN2), mRNA [NM_001103]	GO:0016265 death
LAG3	A_23_P116942	X	X	X	X			X	X				lymphocyte-activation gene 3 (LAG3), mRNA [NM_002286]	GO:0008219 cell death
AXUD1	A_23_P121011	X	X	X									AXIN1 up-regulated 1 (AXUD1), mRNA [NM_033027]	GO:0006915 apoptosis
IL10	A_23_P126735	X	X	X	X	X	X	X	X	X	X	X	interleukin 10 (IL10), mRNA [NM_000572]	GO:0012501 programmed cell death
SOCS2	A_23_P128215	X	X	X	X	X	X	X	X				suppressor of cytokine signaling 2 (SOCS2), mRNA [NM_003	GO:0043066 negative regulation of apoptosis
MPO	A_23_P141173	X	X	X	X	X	X	X	X	X	X	X	myeloperoxidase (MPO), nuclear gene encoding mitochondria	GO:0043069 negative regulation of programmed cell de
BCL2A1	A_23_P152002	X	X	X	X	X	X	X	X	X	X	X	BCL2-related protein A1 (BCL2A1), mRNA [NM_004049]	GO:0042981 regulation of apoptosis
GCH1	A_23_P163079	X	X	X	X			X	X				GTP cyclohydrolase 1 (dopa-responsive dystonia) (GCH1), tra	GO:0043067 regulation of programmed cell death
OSM	A_23_P166408	X	X	X	X								oncostatin M (OSM), mRNA [NM_020530]	GO:0006916 anti-apoptosis
COL4A3	A_23_P170679	X	X	X	X			X	X				collagen, type IV, alpha 3 (Goodpasture antigen) (COL4A3), t	GM:hs_negative_regulation_of_apoptosis
SGK	A_23_P19673	X	X	X	X								serum/glucocorticoid regulated kinase (SGK), mRNA [NM_00	GM:hs_negative_regulation_of_programmed_cell_death
SOCS3	A_23_P207058	X	X	X	X	X	X	X	X				suppressor of cytokine signaling 3 (SOCS3), mRNA [NM_003	
PMAIP1	A_23_P207999	X	X	X	X			X	X				phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1),	
IL4	A_23_P213706	X	X	X	X	X	X	X	X				interleukin 4 (IL4), transcript variant 1, mRNA [NM_000589]	
TNFRSF10C	A_23_P256724	X	X	X	X								tumor necrosis factor receptor superfamily, member 10c, dec	
PHLDA1	A_23_P338912	X	X	X	X								pleckstrin homology-like domain, family A, member 1 (PHLDA	
FOSL2	A_23_P343671	X	X										mRNA; cDNA DKFZp686E0486 (from clone DKFZp686E0486	
PIM1	A_23_P345118	X	X	X	X	X	X	X	X				pim-1 oncogene (PIM1), mRNA [NM_002648]	
FOSL2	A_23_P348121	X	X										FOS-like antigen 2 (FOSL2), mRNA [NM_005253]	
SOCS3	A_23_P351069	X	X	X	X	X	X	X	X				suppressor of cytokine signaling 3 (SOCS3), mRNA [NM_003	
TBX3	A_23_P383819	X	X	X	X	X	X	X	X				T-box 3 (ulnar mammary syndrome) (TBX3), transcript variant	
MMP9	A_23_P40174	X	X	X	X			X	X				matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 9	
IER3	A_23_P42257	X	X	X	X	X	X	X	X	X	X	X	immediate early response 3 (IER3), transcript variant short, m	
CDKN1A	A_23_P59210	X	X	X	X	X	X	X	X				cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), t	
AGTR2	A_23_P62309	X	X	X	X								angiotensin II receptor, type 2 (AGTR2), mRNA [NM_000686]	
BTG2	A_23_P62901	X	X	X	X	X	X	X	X				BTG family, member 2 (BTG2), mRNA [NM_006763]	
IL6	A_23_P71037	X	X	X	X	X	X	X	X				interleukin 6 (interferon, beta 2) (IL6), mRNA [NM_000600]	
PHLDA1	A_23_P76450	X	X	X	X								pleckstrin homology-like domain, family A, member 1 (PHLDA	
IL1B	A_23_P79518	X	X	X	X								interleukin 1, beta (IL1B), mRNA [NM_000576]	
CCL2	A_23_P89431	X	X	X	X	X	X	X	X	X	X	X	chemokine (C-C motif) ligand 2 (CCL2), mRNA [NM_002982]	
TNFAIP3	A_24_P157926	X	X	X	X	X	X	X	X	X	X	X	tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), mR	
GCH1	A_24_P167642	X	X	X	X			X	X				GTP cyclohydrolase 1 (dopa-responsive dystonia) (GCH1), tra	
SOCS2	A_24_P230675	X	X	X	X	X	X	X	X	X			suppressor of cytokine signaling 2 (SOCS2), mRNA [NM_003	
TNFSF14	A_24_P237036	X	X	X	X			X	X		X	X	tumor necrosis factor (ligand) superfamily, member 14 (TNFS	
GADD45B	A_24_P239606	X	X	X	X								growth arrest and DNA-damage-inducible, beta (GADD45B), r	
MCL1	A_24_P319635	X	X	X	X	X	X	X	X	X	X	X	myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), tra	
GJA1	A_24_P55295	X	X	X	X								gap junction protein, alpha 1, 43kDa (connexin 43) (GJA1), m	
CDKN1A	A_24_P89457	X	X	X	X	X	X	X	X				cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), t	
	A_24_P915692	X	X	X	X								pleckstrin homology-like domain, family A, member 1 (PHLDA	
PHLDA1	A_24_P943597	X	X	X	X								pleckstrin homology-like domain, family A, member 1 (PHLDA	
PROK2	A_24_P97342	X	X	X	X	X	X	X	X	X	X	X	prokineticin 2 (PROK2), mRNA [NM_021935]	
SERPINB9	A_32_P38323	X	X	X	X	X	X	X	X				serpin peptidase inhibitor, clade B (ovalbumin), member 9 (SE	

10. Curriculum Vitae

Personal data

Name: Sara Dummler

Date of birth: 1st November, 1978

Place of birth: Pfaffenhofen a.d.Ilm, Germany

Education

2007 – 2012 PhD, Technische Universität München, Freising, Germany
2005 – 2007 PhD, Ludwig-Maximilians-Universität München, Germany
2003 – 2004 Diploma thesis at the Department Biology II Anthropology and Human Genetics. Title: " Vertebrate food webs at the time of the Neolithic transition in Anatolia ".
1998 – 2004 Ludwig-Maximilians-Universität München, Germany
Field of study: Biology
Degree: Diploma
1989 – 1998 Schyren-Gymnasium Pfaffenhofen
Degree: Abitur

Professional experience

2007 – 2011 Employment as Ph.D. Candidate and Research Associate at the German
Heart Centre Munich, Department of Cardiovascular Surgery, Experimental Surgery.
2003 – 2006 Student assistant at the Institute of Genetics, group of Prof. Dr. Michael Boshart, Ludwig-Maximilians-Universität München, Germany
2005 Student assistant at the Department of Prehistoric Archaeology, Philipps-
Universität Marburg, Germany
2004 Student Assistant at the Institute of Anthropology and Human Genetics, Ludwig-Maximilians-Universität München, Germany
2002 Internship at the Max-von-Pettenkofer-Institute, Group of Prof. Dr. Holger
Rüssmann, Ludwig-Maximilians-Universität München, Germany

Attendance at conferences

Poster

Krane M, Dummler S, Dressen M, Hoffmann M, Heller K, Hauner H, Lahm H, Tesche C, Lange R, Bauernschmitt R. Up-regulation of the anti-apoptotic gene MCL-1 in the atherosclerosis-resistant internal thoracic artery. Annual congress of the European Society of Cardiology (ESC) 2009, Barcelona, Spain.

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Dummler S., von den Driesch A., Grupe G. The development of free ranging bird populations in Europe during the Holocene. 6th Congress of the Society for Anthropology (GfA) 2005, Munich, Germany

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International Journal of Cardiology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Identification of an up-regulated anti-apoptotic network in the internal thoracic artery

Markus Krane [a,*,1], Sara Dummler [a,*,1], Martina Dreßen [a], Hans Hauner [b], Micha Hoffmann [c], Dirk Haller [c], Katharina Heller [c], Stephen Wildhirt [a], Bernhard Voss [a], Joachim Grammer [a], Harald Lahm [a], Rüdiger Lange [a], Robert Bauernschmitt [a]

[a] German Heart Centre Munich at the Technische Universität München, Department of Cardiovascular Surgery, Lazarettstrasse 36, 80636 Munich, Germany
[b] Else-Kröner-Fresenius-Centre for Nutritional Medicine at the Technische Universität München, Gregor-Mendel-Str. 2, 85350 Freising-Weihenstephan, Germany
[c] Chair for Biofunctionality, ZIEL-Research Centre for Nutrition and Food Science at the Technische Universität München, Gregor-Mendel-Str. 2, 85350 Freising-Weihenstephan, Germany

ARTICLE INFO

Article history:
Received 18 August 2009
Received in revised form 19 November 2009
Accepted 3 February 2010
Available online xxxx

Keywords:
Atherosclerosis
Intimal hyperplasia
Radial artery
Internal thoracic artery
Anti-apoptosis
Gene expression

ABSTRACT

Background: The radial artery (RA) is known as an atherosclerosis-prone vessel in contrast to the atherosclerosis-resistant internal thoracic artery (ITA). The purpose of the present study was to compare the gene expression profile of these arteries from the same patient in order to identify genes involved in atherogenesis or intimal hyperplasia.
Methods: Paired specimens of RA and ITA ($n=6$) were analyzed by histomorphometry and whole genome microarray. The microarray data underwent pathway analysis to identify biological networks. Laser microdissection (LMD) was used to identify the cellular expression of candidate genes in the intimal or medial layer of the ITA and RA.
Results: Histomorphometric analyses revealed a significantly higher degree of intimal hyperplasia in the RA compared to the ITA. 552 genes were differentially expressed in the ITA and RA. qRT-PCR confirmed a significant up-regulation of six anti-apoptotic genes. p21 (11.8-fold, $p=0.011$), CCL2 (5.4-fold, $p=0.034$), SOCS3 (7.2-fold, $p=0.002$), IER3 (4.1-fold, $p=0.048$), MCL-1 (2.6-fold, $p=0.025$) and IL-6 (17.8-fold, $p=0.046$) were up-regulated in the ITA. LMD confirmed that cells of the intimal layer of the ITA consistently expressed higher levels of all six candidate genes than those of the RA.
Conclusions: Microarray analysis and qRT-PCR identified significantly up-regulated genes in the ITA involved in an anti-apoptotic network. LMD revealed a higher expression of all anti-apoptotic genes in the intimal area of the ITA. These genes may play an important role in protecting the intima of the ITA from developing hyperplasia and atherosclerosis.

1. Introduction

Apoptosis has been identified as a prominent feature of advanced human atherosclerosis plaques affecting the cellularity and integrity of plaques. Recent reports discuss apoptotic mechanisms involved in the process of early atherogenesis [1,2]. Throughout the process of atherosclerosis, both smooth muscle cells (SMCs) and endothelial cells (ECs) undergo programmed cell death [3]. The general understanding of the apoptotic process in atherogenesis as well as the anti-apoptotic and athero-protective mechanisms in some arteries resistant to intimal hyperplasia and atherosclerosis remain unclear at present.

Intimal hyperplasia occurs as a consequence of physiological stimuli, constituting an attempt by the tissue to maintain normal conditions of flow, wall tension, or both [4] and is typically associated with the initiation of atherosclerosis. Regions of the intima with adaptive increases in thickness differ functionally from adjacent, thinner regions. The turnover of ECs and SMCs is increased [5] and excessive lipoprotein in the plasma tends to accumulate preferentially in the hyperplastic intima, causing atherosclerosis [6].

Intimal hyperplasia is particularly characterized by proliferation and abnormal migration of SMCs from the media into the intima [7,8]. Later these SMCs deposit an extracellular matrix [7]. Bochaton-Piallat et al. figured out that apoptosis of SMCs in the aorta of rats is an important mechanism in the regulation of intimal thickening evolution after endothelial denudation [9]. The internal thoracic artery (ITA) is known as an atherosclerosis-resistant artery, as opposed to the atherosclerosis-prone radial artery (RA). Atherogenesis and intimal hyperplasia occur more frequently in the RA (incidence 10%) than in the ITA (incidence <1%) [10]. Both arteries

* Corresponding authors. Krane is to be contacted at Tel.: +49 89 12184111; fax: +49 89 12184113. Dummler, Tel.: +49 89 12183505; fax: +49 89 12183513.
E-mail addresses: krane@dhm.mhn.de (M. Krane), dummler@dhm.mhn.de (S. Dummler).
[1] These authors have equally contributed.

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doi:10.1016/j.ijcard.2010.02.003

show histomorphological differences with a thicker media in the RA (1.7-fold thicker than in the ITA) containing SMC arranged in several tight layers [11,12]. Furthermore, the elastic laminae of the RA are monolayers with multiple fenestrations in contrast to no fenestrations in the ITA [13].

The differences in morphology of the RA may be responsible for its increased vulnerability for intimal hyperplasia. However, the underlying molecular mechanisms of this process are unclear at present.

Based on the elevated occurrence of intimal hyperplasia in the RA opposed to the atherosclerosis-resistant ITA, the purpose of the study was to determine significant differences in the gene expression profile between the ITA and the RA from the same patient by whole genome microarray analysis and validation by qRT-PCR. In addition, laser microdissection (LMD) technique was used to provide evidence that cells of the intimal or medial layer are responsible for the elevated RNA expression of our genes of interest.

2. Methods

2.1. Tissue samples

Tissue samples of the distal ITA and the distal RA were analyzed from six patients who underwent coronary artery bypass grafting (CABG) (Table 1). Each sample was immediately divided into two parts at the time of surgery: one was fixed in 4% PBS-buffered formalin (pH 7.4) and processed for morphometry, while the other part was frozen in liquid nitrogen and stored at −75 °C for RNA analysis. The study was approved by the ethical committee at the Technical University of Munich, Medical Faculty (file number 1588/06) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and each patient signed an informed consent for inclusion in the study.

2.2. Morphometry

Sections of 3 and 4 µm were cut from paraffin-embedded tissue specimens and stained with hematoxylin and eosin (Supplementary Fig. 1). In this study, three morphometric parameters were used to describe the degree of intimal thickening (Table 2) [6]. Histomorphometric data were obtained using the Image-Pro® Software (Media Cybernetics, Inc., MD).

2.3. Gene expression and pathway analyses

From each donor a pair of samples from the RA and the ITA was available and RNA was isolated using standard RNA extraction protocols (Trizol, Sigma-Aldrich, Steinheim, Germany). RNA quality was assessed by the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Böblingen, Germany) and was selected for the linear T7-based amplification (according to the manufacturer's protocol). The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). A Significance Analysis of Microarrays (SAM) was performed in order to identify genes which were consistently differentially regulated in all donors. The resulting discriminatory genes were provided in all clustered row-normalized heat maps. The list of genes significantly up- or down-regulated in the RA samples in comparison to the ITA samples were tested for a significant enrichment of annotation using the proprietary *TreeRanker* software (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). To identify the biological context of the discriminatory genes, pathway analysis was performed by using the *PathwayArchitect*™ software (Stratagene, La Jolla, CA) to generate molecular networks (Miltenyi Biotec GmbH).

Table 1
Basic clinical characteristics of patients.

Specimen no.	1	2	3	4	5	6
Age (years)	58	47	56	66	81	65
Body weight (kg)	69	108	80	82	79	85
Height (cm)	172	180	165	177	176	178
BMI	23.32	33.33	29.41	26.17	25.50	26.83
Diabetes mellitus	Yes	No	No	No	No	Yes
Hypercholesterolemia	No	No	Yes	No	Yes	No
Art. hypertension	Yes	Yes	Yes	Yes	No	Yes
Family history of CAD	Yes	Yes	No	No	No	No
EF (%)	69	51	50	75	Unknown	62
Case history of nicotine abuse	Yes	Yes	Yes	No	No	Yes
No. of bypass grafts	3	3	3	2	2	3

BMI = body mass index; EF = ejection fraction; CAD = coronary artery disease.

Table 2
Intimal thickening in the ITA and RA.

	ITA	RA	*p* value
% LN	11.56 ± 7.40	25.17 ± 11.83	0.025
ITI	0.13 ± 0.05	0.20 ± 0.09	0.045
IMR	0.19 ± 0.11	0.46 ± 0.18	0.016

%LN = 100 × intimal area / lumen and intimal area (percentage of luminal narrowing), ITI = intimal area / medial area (intimal thickness index), IMR = maximal width of intima / maximal width of media (intima-to-media ratio). Data represent mean values ± SD.

2.4. mRNA quantification by real-time qRT-PCR

The suggested anti-apoptotic genes cyclin-dependent kinase inhibitor 1A (CDKN1A, synonym p21), Chemokine (C-C motif) ligand 2 (CCL2, synonym MCP-1), suppressor of cytokine signalling 3 (SOCS3), immediate early response 3 (IER3), myeloid cell leukemia sequence 1 (MCL-1), interleukin-10 (IL-10) and interleukin-6 (IL-6) were validated by qRT-PCR. Specific primer pairs (Supplementary Table 1) were designed for each gene using Primer3 software (http://www.frodo.wi.mit.edu/) and specificity was re-verified using the standard nucleotide basic local alignment search tool (BLASTn) provided by the National Centre of Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Primers were tested for their ability to generate single products of correct sizes by melting curve analysis and agarose gels. Thermal cycling was performed in a Light Cycler 1.5 (Roche Molecular Biochemicals, Mannheim, Germany) using the QuantiTect® SYBR® Green one-step RT-PCR kit (Qiagen, Hilden, Germany). A five-step experimental protocol was used (Supplementary Table 2). The crossing points (CP values) were determined for each transcript applying the Second Derivate Maximum Method using LightCycler Software 3.5 (Roche Molecular Biochemicals).

2.5. Laser microdissection (LMD)

The Leica LMD6000 (Leica Microsystems, Wetzlar, Germany) was used according to the manufacturer's instructions. Formalin-fixed paraffin-embedded (FFPE) tissue samples were cut in 5-µm-thick sections on a microtome with a disposable blade and collected onto PET UV-absorbing membrane slides (RNAse free, Leica Microsystems). Sections were deparaffinized by two changes of xylene for 3 min and rehydrated sequentially in 100% (2 min), 96% (2 min) and 70% ethanol (1 min) in a Leica ST5020 Multistainer (Leica Microsystems).

For qRT-PCR analyses areas of ~1000 cells were microdissected from the arterial intima and media of ITA and RA from four of the six patients (patient number three to six). LMD could not be performed with samples of patient one and two due to a limited sample mass in these patients. Using a relatively constant number of cells for LMD, isolation of cells from each part revealed comparable quantities of RNA. Total RNA was extracted using the miRNeasy® FFPE kit (Qiagen, Hilden, Germany) and reverse-transcribed in a final volume of 20 µl using random hexamer primer and the SuperScript® II Reverse Transcription kit (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. As formalin-fixation is known to cause RNA fragmentation additional primer pairs which amplify shorter fragments were designed for each of the anti-apoptotic genes using Primer3 software (Supplementary Table 3).

2.6. Statistics

The differences in the degree of intimal thickening and atherosclerotic sensitivity in the two arteries are expressed as mean value ± standard deviation (SD) using the Mann–Whitney–Wilcoxon test for unpaired variables. *P* values less than 0.05 were considered significant and samples were, therefore, selected for microarray analysis.

Statistical analysis of gene expression data was performed considering RT-PCR efficiencies for each gene (calculated from standard curves) and running pair-wise fixed reallocation randomization tests® using the relative expression software tool REST© (http://www.wzw.tum.de/gene-quantification/). *P*-values lower than 0.05 were considered statistically significant. Details of the procedure are outlined in Pfaffl et al. [14].

3. Results

3.1. Morphometric analysis showed a significantly increased degree of intimal hyperplasia in the RA

The ITA and the RA of six male patients with a mean age of 62.2 ± 11.5 years were morphologically analyzed for the presence of intimal hyperplasia. Detailed patient characteristics are given in Table 1. The degree of the intimal thickening was analyzed by applying three morphometric parameters: (1) the mean percentage of luminal narrowing (%LN), (2) the intimal thickness index (ITI) and (3) the intima-to-media ratio (IMR). All three values were significantly increased in the RA compared to the ITA (Table 2).

Please cite this article as: Krane M, et al, Identification of an up-regulated anti-apoptotic network in the internal thoracic artery, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.02.003

3.2. Microarray and pathway analyses reveal activation of an anti-apoptotic network

Having confirmed differences in the degree of intimal hyperplasia between the ITA and RA we extracted total RNA and performed a comparative genome wide expression analysis. The RNA Integrity Number (RIN) was >6 for all selected samples (Supplementary Fig. 2). Comparison of the RA to the ITA samples revealed 1.35% differentially expressed genes. Our analysis yielded 418 up-regulated genes in the ITA and 134 up-regulated genes in the RA with a False Discovery Rate (FDR) of 5.3%. Significantly up- or down-regulated genes were tested for an enrichment of annotations using the proprietary *TreeRanker* software to add structure to genomic data in the formed pathways. The RA samples showed up-regulation of single genes in several categories. However, no molecular networks and pathways could be generated. The up-regulated genes in the ITA showed annotations in several categories such as proliferation, cytokines and chemotaxis. In particular, 35 genes with potential anti-apoptotic activity (Supplementary Table 4) were annotated with high significance. The anti-apoptotic pathway generated from this cluster of genes (Fig. 1) was selected for further analysis.

3.3. Validation of the anti-apoptotic network by real-time qRT-PCR

We used qRT-PCR to analyze the expression of the selected candidate genes MCL-1, IER3, SOCS3, p21, IL-6, IL-10 and CCL2 on RNA preparations of tissue samples used for the microarray analysis by qRT-PCR. Significant up-regulation was observed for MCL-1 (2.6-fold, $p=0.025$), IER3 (4.1-fold, $p=0.048$), CCL2 (5.4-fold, $p=0.034$), SOCS3 (7.2-fold, $p=0.002$), p21 (11.8-fold, $p=0.011$) and IL-6 (17.8-fold, $p=0.046$) in the ITA compared to the RA (Fig. 2). Thus, qRT-PCR analysis confirmed the results from the microarray analysis in 6 of the identified 11 genes of the anti-apoptotic pathway. In contrast, an elevated level of IL-10 expression as suggested by the microarray analysis could not be validated by qRT-PCR (1.47-fold, $p=0.55$).

3.4. Elevated expression of the anti-apoptotic network in cells of the intimal layer

LMD of cells from the intimal and medial layers of FFPE-sections from RA and ITA of 4 of the 6 patients was performed. Fig. 3 shows representative sections of each layer before (Fig. 3 panels A) and after (Fig. 3 panels B) microdissection. For gene expression analysis, cells from each layer, artery and patient were dissected on independent caps.

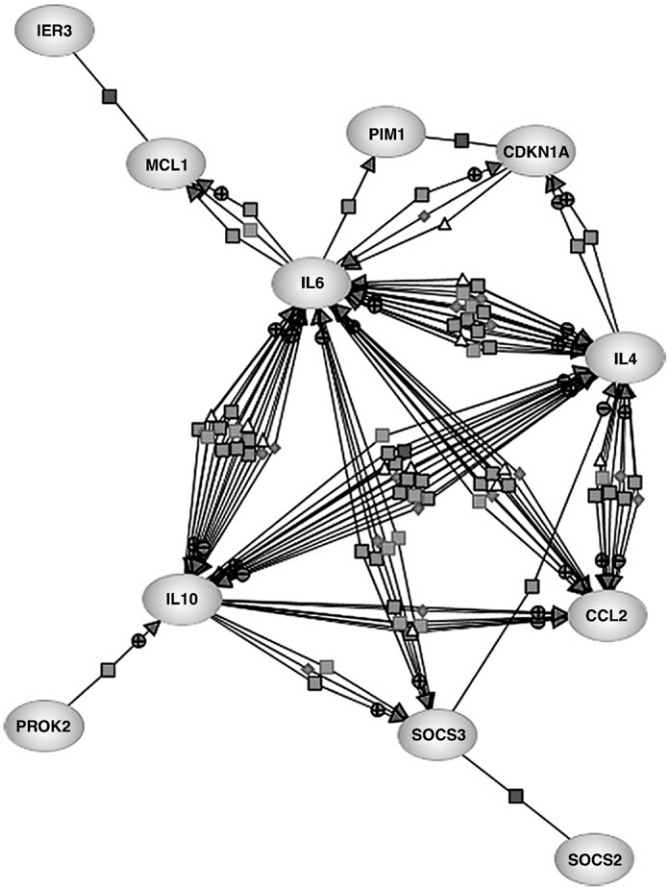

Fig. 1. Biological interaction network of genes related to anti-apoptosis as suggested by the *PathwayArchitect* Software.

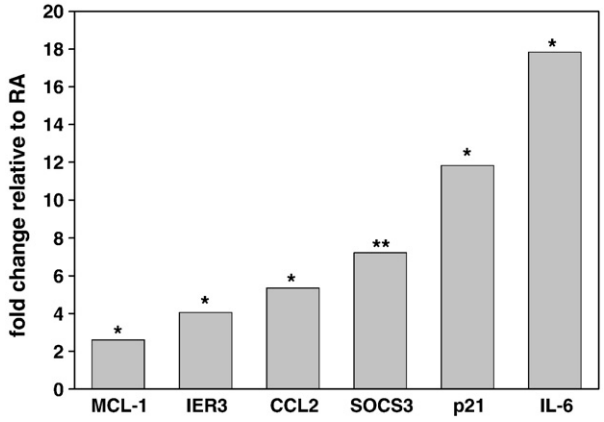

Fig. 2. Anti-apoptotic genes show an elevated expression in the atherosclerosis-resistant ITA. Ratios of differentially expressed genes of the anti-apoptotic network (MCL-1, IER3, SOCS3, p21, IL-6, and CCL2) in the ITA relative to the RA. $^{*}p<0.05$; $^{**}p<0.01$.

The house-keeping gene β-actin served as reference. Firstly, we compared the expression between corresponding layers of the two arteries. Expression analysis of the six identified anti-apoptotic genes revealed the up-regulation of MCL-1 (2.8-fold), IER3 (8.98-fold), SOCS3 (7.1-fold), p21 (6.2-fold), IL-6 (4.1-fold) and CCL2 (7.6-fold) in the intimal layer of the ITA compared to the RA (Fig. 4A). Likewise, the medial area of the ITA in comparison to the medial area of the RA showed an up-regulation of the anti-apoptotic genes IER3 (7.0-fold), SOCS3 (6.6-fold), p21 (14.5-fold), IL-6 (4.4-fold) and CCL2 (17.6-fold). MCL-1 (1.4-fold) showed no differential expression in the media of the ITA and the RA (Fig. 4B). These differences were statistically significant for p21 and IER3 when comparing the medial layer of both arteries.

Comparing the intimal and the medial area of the ITA gene expression data showed a significant up-regulation of MCL-1 (Fig. 5A). In the intimal area of the RA the expression of MCL-1, p21 and CCL2 were significantly enhanced when compared to the medial area (Fig. 5B). For IER3, SOCS3 and IL-6 the difference was not significant most probably due to the limited number of samples. In summary, the expression of all candidate genes was consistently higher in the intimal layer of both arteries.

4. Discussion

Previous studies comparing atherosclerotic and non-atherosclerotic arteries described apoptosis-related mechanisms [15,16]. These studies were limited by the fact that they compared vessels obtained from different patients. The objective of this study was to identify molecular signatures or candidate genes which discriminate the atherosclerosis-resistant ITA from the atherosclerosis-prone RA, both derived from the same individual. To our knowledge, this is the first report of a gene expression profile revealing differences between the ITA and the RA in the same patient.

4.1. Morphometric analysis

Prior to any molecular analysis we first determined different morphometric parameters commonly used to define intimal changes. Our morphometric data revealed a significantly increased IMR, ITI and %LN in the six RA samples compared to the ITA samples suggesting a higher grade of intimal hyperplasia in the RA. These findings are in agreement with other studies showing a higher extent of intimal hyperplasia and atherosclerotic plaque formation in the RA compared to the ITA [13,17]. Our results for the ITI and the %LN correspond well with the results of Ruengsakulrach et al. who examined the comparative histopathology, morphometry, and risk factors for the development of intimal hyperplasia and atherosclerosis in the ITA and RA from 150 patients undergoing CABG [4]. They reported similar values for the morphometric parameters ITI and %LN in the ITA ($ITI=0.15\pm0.82$, $\%LN=11\pm6$) and in the RA ($ITI=0.26\pm0.22$, $\%LN=21\pm11$). Only the IMR ($ITA=0.36\pm0.37$, $RA=0.85\pm1.38$) diverged from our results possibly due to patients with an advanced atherosclerotic lesion type V. Malhotra et al. also reported an ITI of

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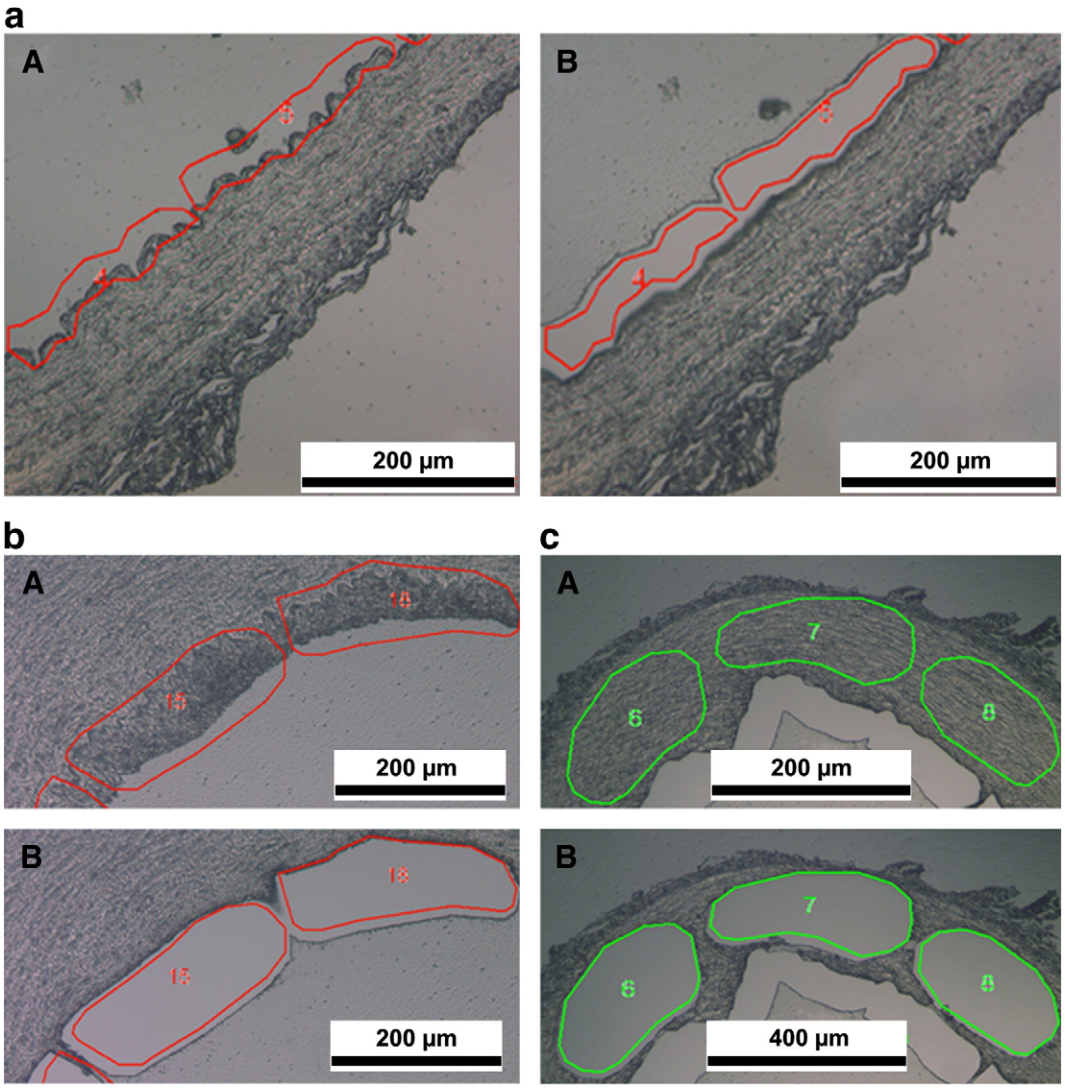

Fig. 3. Laser microdissection of cells from the intimal and medial layer of FFPE-sections from patients. a: intima of the ITA, b: intima of the RA, c: medial layer of the ITA. The tissue section is shown before (A) and after (B) removal of the selected cells.

0.05 ± 0.1 for the ITA in 25 patients [18] which is comparable to our results. Thus, our samples of the RA showed a significantly increased degree of intimal hyperplasia compared to the ITA and these results correspond well with previously reported data.

4.2. Network of anti-apoptotic genes

The results of the microarray analysis and the relative quantification by RT-PCR confirmed a significant up-regulation of MCL-1, p21, IER3, SOCS3, IL-6 and CCL2 in the ITA compared to the RA in all six patients. LMD revealed an elevated mRNA concentration of our genes of interest in the intima of the ITA compared to the intima of the RA. Furthermore, comparing the relative expression levels between the intima and the media of the same artery, we could also show a consistently increased gene expression of our genes of interest in the intimal layer of both the ITA and the RA.

The Bcl-2 homologue MCL-1 is known to block apoptosis as well as to regulate the cell cycle. The N-terminus of MCL-1 has two major regulatory roles, namely regulating co-ordinately the mitochondrial (anti-apoptotic) and the nuclear (anti-proliferative) functions of MCL-1 [19]. The anti-apoptotic property of MCL-1 seems to be associated with a mitochondrium-related mechanism by regulating the mitochondrial outer membrane permeabilization for pro-apoptotic factors and with nuclear functions of anti-proliferative character by delaying cell cycle progression [20,21]. An increased transcription of the anti-apoptotic gene Bcl-2 was demonstrated via the activation of the phosphoinositide 3-kinase (PI-3K) pathway. Bcl-2 can be induced by protein kinase $C\in$ to form a signalling complex and to act co-operatively with AKT to protect human vascular endothelial cells against apoptosis and thus atherogenesis [22].

The transcription of IER3, also known as IEX-1, is under the control of several transcription factors, which are capable of manipulating mutually exclusive processes, for instance, cell survival vs. cell death or cell division vs. cell cycle arrest, depending on the cell types and the number and strength of stimuli. IEX-1 was found to be constitutively expressed in native non-injured vessels but was barely detectable in intimal hyperplastic lesions produced by balloon injury and endothelial denudation [23]. Schulze et al. [24] postulated that the induction of IEX-1 functions as part of a negative feedback mechanism limiting the local vascular response and the proliferation of vascular SMCs (VSMCs) in atherosclerosis and after vascular injury. The functional effects of IEX-1 *in vivo* have been investigated by performing carotid artery mechanical injury and endothelial denudation in LDL receptor deficient mice followed by intraluminal injection of adenoviral vectors for overexpression of IEX-1. The vascular up-regulation of IEX-1 strongly inhibited

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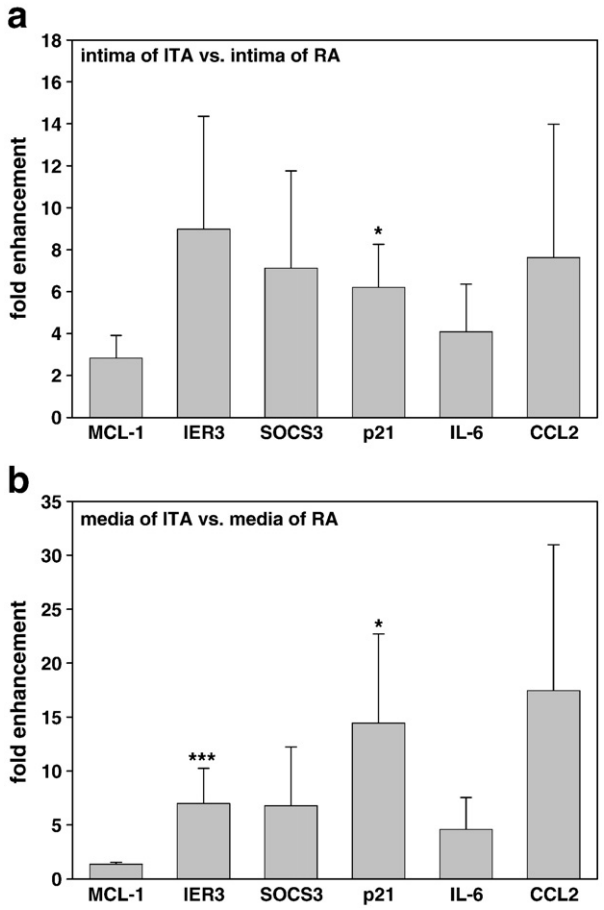

Fig. 4. Anti-apoptotic genes are highest expressed in the intimal layer of the ITA. Gene expression analysis of the anti-apoptotic genes on laser-microdissected FFPE-sections by qRT-PCR. a: intima of ITA vs. intima of RA, b: media of ITA vs. media of RA. $^{*}p<0.05$; $^{***}p<0.005$.

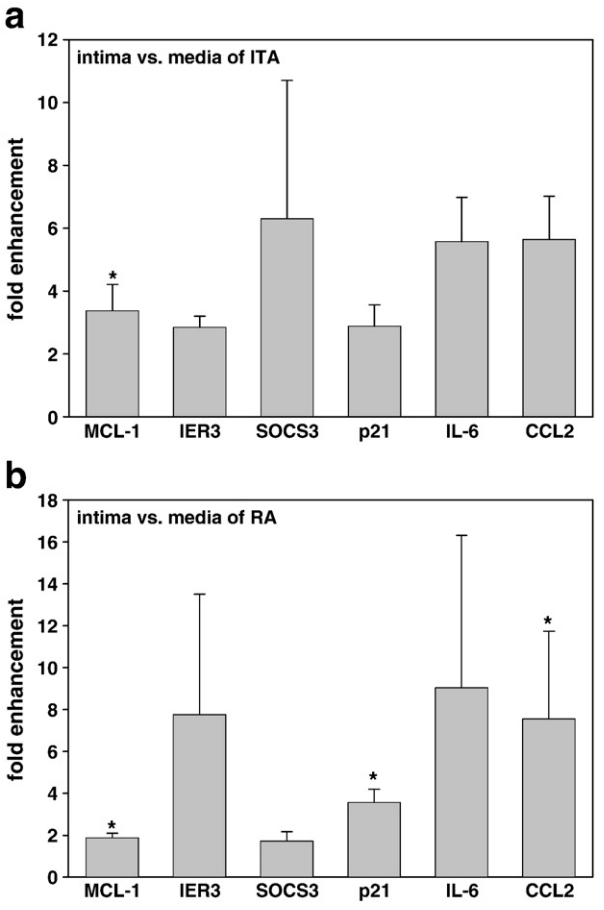

Fig. 5. Comparison of anti-apoptotic gene expression in intimal and medial layers of individual arteries. Gene expression analysis of the anti-apoptotic genes on laser-microdissected FFPE-sections by qRT-PCR. a: intimal vs. medial layer of the ITA, b: intimal vs. medial layer of the RA. $^{*}p<0.05$.

neointima formation after vascular injury *in vivo* compared to controls [24].

SOCS3 encodes a member of the signal transducers and activators of transcription (STAT)-induced STAT inhibitor (SSI, cytokine-inducible negative feedback inhibitors of cytokine signalling via the JAK/STAT pathway) family, also known as suppressor of cytokine signalling (SOCS). The protein encoded by SOCS3 can bind to Janus kinase 2 (JAK2), and seems to inhibit JAK catalytic activity [25]. Seki et al. [26] examined the role of the JAK/STAT pathway in vascular remodelling after balloon-injured rat carotid arteries. They found elevated levels of phosphorylated STAT3 in the medial and neointimal VSMCs of the injured arteries, whereas no STAT3 expression could be detected in the intact arteries. After local treatment with a JAK2-specific inhibitor, STAT3 phosphorylation and neointimal VSMC replication were inhibited and neointima formation was reduced in the injured arteries. In a subsequent study, Shibata et al. [27] inhibited endogenous STAT3 activation which resulted in reduced proliferation of VSMCs and a 40% reduction in the intima/media area ratio of the arteries. Otherwise, deletion of the SOCS3 gene results in hyperactivation of STAT3 and induces apoptosis [28], suggesting that SOCS3 is a critical regulator being capable of converting from a pro-apoptotic to an anti-apoptotic messenger [25].

The gene p21 is known to affect cell cycle progression and is likely to play a role in limiting arterial cell proliferation [29]. Several studies have described a reduction of neointima formation by the overexpression of p21 using adenoviral vectors [30,31]. Yang et al. reported growth arrest for both ECs and VSMCs through the ability of p21 to inhibit progression of the cell cycle [29].

In addition, our data also suggest an enhanced expression of IL-6 and CCL2 in the atherosclerosis-resistant ITA. At first hand this appears to be surprising as both molecules are potent mediators of inflammation and attract monocytes during early atherosclerosis [32,33]. However, both factors also can antagonize apoptosis by up-regulation of anti-apoptotic proteins such as cFLIP (cellular FLICE-like inhibitory protein), Bcl-2 or Bcl-X [34] or through activation of p38 MAPK and subsequent stimulation of PKCδ activity [35]. Furthermore, the activity of CCL2 and subsequent production of IL-6 mediated survival of fibroblasts in lung tissue [36]. Thus, it is tempting to speculate that the combined up-regulation of both genes may lead to similar consequences in the atherosclerosis-resistant ITA.

The early phase of atherogenesis is defined by the endothelial dysfunction and inflammation with prominent lipid retention. This pro-inflammatory microenvironment predominantly provokes a proliferative response for VSMCs, followed by intimal migration and neointima formation. In addition, EC apoptosis is apparently associated with the development of EC dysfunction and early atherosclerotic lesions show an extensive EC turnover with dysfunctional endothelial cells. Thus, atherogenesis in the early phase is supported by a pro-apoptotic stimulation of ECs and an anti-apoptotic and proliferative stimulation of SMCs [37].

Our results underline this hypothesis of an anti-apoptotic network which is differentially and increasingly expressed particularly in the intima of the ITA thereby protecting the vessel against intimal hyperplasia and atherosclerosis.

While our results strongly suggest the existence of an up-regulated anti-apoptotic network we also have to mention some limitations. The

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sample size is rather small due to the difficulty to include appropriate samples. Furthermore, clinical and epidemiological parameters such as diabetes or age might have an influence on gene expression. After all the inclusion of paired samples from the same patient ensures that both arteries are equally affected which should at least reduce such an effect. Nevertheless, it is desirable to confirm these results with a larger series of samples.

In conclusion, six ITA samples and RA samples from the same patients, analyzed by gene expression profiling revealed 418 significantly up-regulated genes in the ITA and 134 significantly up-regulated genes in the RA. The identified anti-apoptotic genes MCL-1, p21, IL-6, CCL2, IER3 and SOCS3 were verified by qRT-PCR. Laser microdissection of intimal and medial parts of ITA and RA revealed a stronger expression of the anti-apoptotic genes in the intimal EC layer compared to the medial SMC layer and a higher expression in the intima of the ITA versus the radial intimal layer. Therefore, our data permit the hypothesis that anti-apoptotic mechanisms may protect the intimal layer of the ITA from intimal hyperplasia and atherogenesis.

Acknowledgements

This work was supported by institutional and departmental sources from the Department of Cardiovascular Surgery of the German Heart Center. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [38].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at DOI: 10.1016/j.ijcard.2010.02.003.

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Pulsatile *ex vivo* perfusion of human saphenous vein grafts under controlled pressure conditions increases MMP-2 expression

Sara Dummler[1*†], Stefan Eichhorn[1*†], Christian Tesche[1], Ulrich Schreiber[1], Bernhard Voss[1], Marcus-André Deutsch[1], Hans Hauner[2], Harald Lahm[1], Rüdiger Lange[1] and Markus Krane[1]

* Correspondence: dummler@dhm.mhn.de; stefan.eichhorn@dhm.mhn.de
[1]German Heart Center Munich at the Technische Universität München, Department of Cardiovascular Surgery, Lazarettstrasse 36, D-80636 Munich, Germany
Full list of author information is available at the end of the article

Abstract

Background: The use of human saphenous vein grafts (HSVGs) as a bypass conduit is a standard procedure in the treatment of coronary artery disease while their early occlusion remains a major problem.

Methods: We have developed an *ex vivo* perfusion system, which uses standardized and strictly controlled hemodynamic parameters for the pulsatile and non-static perfusion of HSVGs to guarantee a reliable analysis of molecular parameters under different pressure conditions. Cell viability of HSVGs (n = 12) was determined by the metabolic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) into a purple formazan dye.

Results: Under physiological flow rates (10 mmHg) HSVGs remained viable for two weeks. Their exposure to arterial conditions (100 mmHg) was possible for one week without important reduction in viability. Baseline expression of matrix metalloproteinase-2 (MMP-2) after venous perfusion (2.2 ± 0.5, n = 5) was strongly up-regulated after exposure to arterial conditions for three days (19.8 ± 4.3) or five days (23.9 ± 6.1, $p < 0.05$). Zymographic analyses confirmed this increase on the protein level. Our results suggest that expression and activity of MMP-2 are strongly increased after exposure of HSVGs to arterial hemodynamic conditions compared to physiological conditions.

Conclusion: Therefore, our system might be helpful to more precisely understand the molecular mechanisms leading to an early failure of HSVGs.

Keywords: Atherosclerosis, Bypass-Surgery, MMP, Perfusion, Pulsatile flow, vein graft

Background

Coronary artery bypass grafting (CABG) using venous grafts is a standard procedure in the treatment of advanced coronary artery disease. However, vein graft occlusion implanted in an arterial pressure environment is still a major problem [1]. Approximately 15 to 20% of human saphenous vein grafts (HSVGs) occlude within one month and 25% within the first year. Ten years after CABG about 50% of the HSVGs are occluded and 25% have been severely stenosed [2-6]. Early changes in vein grafts include endothelial disruption leaving the graft vulnerable to thrombotic incidents and smooth muscle cell (SMC) migration and proliferation from the media into the intima

within the first week after grafting [1,7]. The vein graft intimal thickening and remodeling occurs as an adaptation to increased wall stress and arterial flow with up to 15% of graft stenosis during the first year [8]. Under physiological conditions human saphenous veins are exposed to low pressure conditions (~5-10 mmHg), a non-pulsatile flow and a shear stress of 1-6 dyne/cm^2 [9]. After grafting and implantation into the coronary artery system the graft must support higher pressure conditions (~60-140 mmHg), a pulsatile flow and a shear stress range of 10-70 dyne/cm^2 during the cardiac cycle [9,10]. Beyond the first year after bypass surgery the development of graft atheroma and accordingly atherosclerotic vein graft stenosis is the dominant process underlying the failure of HSVGs [1,11]. Formation and evolution of atherosclerotic plaques are associated with variations in matrix metalloproteinase (MMP) expression. The gelatinases play a central role in matrix degeneration and SMC migration, a process which substantially contributes to vein graft failure. The involvement of different MMPs in vascular remodeling has been shown [12-14] whereas little is known about the specific role of gelatinases in HSVGs. While MMP-2 is either absent or only present at low levels in normal veins, its expression becomes elevated after graft implantation which may be a response to injuries during graft preparation or the exposure to the arterial environment [8]. It is generally accepted that the arterial mechanical environment plays a role in vein graft failure, yet the specific mechanical conditions and biological mechanisms have not been completely understood. Vessels cultured under static conditions have been widely used to study effects of pre-existing intimal hyperplasia (IH) [15]. Berceli et al. used a rabbit model to analyze intimal changes and MMP gene and protein expression after bilateral common carotid interposition vein grafting with defined regions of different wall shear [16]. The group of Patterson has used HSVGs in organ culture under static conditions or perfusion for seven days with the restriction of shear force calculation and the differentiation just between low-flow and high-flow conditions [17]. Compared to the animal model of Berceli et al. the *ex vivo* perfusion system of Patterson et al. has a nonpulsatile hemodynamic environment, no blood-surface interaction and potential problems with delivery of nutrition or gas. Gusic and colleagues investigated the role of the mechanical environment in vein remodeling in a higher developed *ex vivo* perfusion system with a main focus on medial and intimal growth in the perfused veins. They ran their perfusions system with five different *ex vivo* hemodynamic environments and showed that pressure and shear stress act independently to regulate vein remodeling [7]. Yet, their study had the limitation of unstable pressure profiles during the course of the experiment. In the present study we have developed an *ex vivo* perfusion system which can be used to perfuse HSVGs with tightly controlled, steady and standardized perfusion profiles. We have defined the viability time course of perfused HSVGs exposed to arterial and venous perfusion profiles. In addition, we provide evidence that our system is suitable to detect alterations of molecular markers such as MMP-2 as a consequence of preparative injury or increased arterial perfusion pressure.

Methods

Tissue Preparation

Nonvaricose HSVGs were obtained from 35 patients (mean age 71.4 ± 7.7 years; nine females, 26 males) undergoing CABG surgery in the German Heart Center Munich.

The endoscopically harvested vein grafts were kept in autologous blood at room temperature until implant. One part of the graft was immediately stored in Ringer solution on ice and transferred from the operating room to the laboratory. One small piece was directly snap-frozen in liquid nitrogen and stored at -80°C until further use as unperfused control tissue. This piece served as a reference to determine relative gene expression. The other part of the vein was mounted into the perfusion device as described. The procedure was acknowledged by the local ethical committee (Ethikkommission der TU München, Project no. 1588/06).

Ex vivo perfusion system

The circuit of the perfusion system is driven by a roller pump ISMATEC S2 (Wertheim, Germany) producing a pulsatile and non-static flow. All silicon tubings and the vessel chamber are sterilized prior to use. The vessel mounting procedure is carried out under a biological safety cabinet (NuAire, Plymouth, MN). Constant pressure conditions are maintained using a syringe pump (MC Medizintechnik GmbH, Alzenau, Germany). The entire system is placed into a styrofoam-isolated chamber to maintain a constant temperature of 37°C. Disposable pressure sensors (DPT-9300, Codan Critical Care, Forstinning, Germany) are placed on both sides of the vessel chamber to permanently monitor and facilitate the control of pressure conditions of the circuit. All functions and settings are controlled by a PC with a program written in java. Pressure is controlled by a PID-algorithm, data are logged continuously.

Perfusion of human saphenous vein grafts

HSVGs were fixed in the perfusion device by suture ligation (Ethibond Vicryl 3-0, Ethicon GmbH, Norderstedt, Germany) and adjusted to a length matching the *in vivo* conditions. Total time from operating room to perfusion was less than one hour. The perfusion medium was DMEM/Ham's F-12 (PAA, Marburg, Germany) supplemented with 10% FCS, 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Veins were perfused with venous conditions (flow: 5 ml/min, 10 mmHg, n = 12) or with arterial conditions (flow: 50 ml/min, 100 mmHg, n = 12) for various time periods. At the end of each experiment vein ends were discarded. The other part of the vein was snap-frozen in liquid nitrogen and stored at -80°C until further use. In long-term experiments the medium was replaced every two days. The pH of the medium remained stable within this period.

Determination of viability of vein grafts and histology

To verify tissue viability, a staining with MTT (Sigma, Munich, Germany) was performed. In the presence of metabolically active viable cells the yellow MTT is converted into a water-insoluble purple formazan product due to reduction by mitochondrial dehydrogenases and other cellular enzymes [18,19]. MTT was stored as a stock solution (5 mg/ml in PBS) at -20°C. Short segments of veins (n = 12) were incubated in MTT diluted in serum-free medium to 0.5 mg/ml for one hour at 37°C. To analyze potential degenerative changes in perfused vessels, sections of formalin fixed and paraffin-embedded samples were analyzed after a conventional hematoxylin/eosin staining.

Quantitative RT-PCR analysis

Frozen tissue pieces were minced using a Precellys24 lysis and homogenization system (Peqlab, Erlangen, Germany) and total RNA was extracted using Trifast according to the manufacturer's recommendation (Peqlab). All RNA preparations were digested with DNase I prior to cDNA synthesis using Omniscript RT kit (Qiagen, Hilden, Germany). One μl of cDNA was amplified on a LightCycler 1.5 thermo cycler (Roche Diagnostics, Mannheim, Germany) using the QuantiTect SYBR Green Kit (Qiagen) and BSA (0.5 mg/ml) in a final volume of 20 μl. All primers were used in a final concentration of 0.5 μM. The following primers were used: β-actin forward 5' CCA ACC GCG AGA AGA TGA 3', β-actin reverse 5' CCA GAG GCG TAC AGG GAT AG 3', MMP-2 forward 5' TGC TGG AGA CAA ATT CTG GA 3', MMP-2 reverse 5' GAT GGC ATT CCA GGC ATC 3'. They amplify fragments of 96 and 90 bp, respectively. After an initial activation of *Taq* polymerase for 15 min at 95°C specific products were amplified during 40 cycles using the following conditions: 15 sec at 94°C (denaturation), 20 sec at 60°C (annealing) and 20 sec at 72°C (elongation). The relative expression levels of MMP-2 in individual samples were calculated in relation to the expression of the β-actin housekeeping gene. To compare independent samples the ratios of MMP-2/β-actin were calculated.

Zymography

MMP-2 protein activities were evaluated by a standard gelatine zymography. Briefly, 100 mg of frozen HSVG tissue were homogenized in ice cold zymogram buffer (150 mM NaCl, 1 μm $ZnCl_2$, 1.5 mM NaN_3, 20 mM $CaCl_2$, 0.01% Triton X-100, 10 mM cacodylic acid, pH 5.0). Samples were centrifuged at 4°C for 10 min at 20.000 × *g*. The supernatant containing proteins was removed and stored at -80°C until further use. Ten μg of extracted protein were mixed with zymogram loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue) and separated in 15% SDS-PAGE gels containing 1 mg/ml type A gelatine from porcine skin (SIGMA-Aldrich, Taufkirchen, Germany). To renature proteins, gels were washed two times in 2.5% Triton X-100 for 15 min at room temperature and subsequently incubated in developing buffer, pH 7.5 (200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 0.02% Brij-35) overnight at 37°C. Gels were stained with 0.5% Coomassie Blue R250 in 40% methanol/10% acetic acid for 15 min and destained in 40% methanol/10% acetic acid until clear bands of lytic activity appeared. The reaction was stopped by transfer of gels in aqua bidest. Gelatinolytic activity was quantified using ImageJ software (version 1.43 u, National Institute of Health). The pixel intensities of bands within each gel were normalized against the respective control of unperfused venous tissue.

Statistical analysis

For the analysis of gene expression levels and MMP-2 gelatinolytic activity the comparison was made using the unpaired Student's *t*-test. Differences in the vessel viability were calculated using the Mann-Whitney U-Test. Differences were considered to be significant at values of $p < 0.05$.

Results

Establishment of the ex vivo perfusion system

Twenty four veins from twenty three patients were used for the *ex vivo* perfusion experiments to establish and proof the reliability of the system. The veins were fixed on tapered conical metal adapters with circular striae to ensure a tight fit of the grafts throughout the whole experiment (Figure 1A). All components used in the vessel chamber are biocompatible (PEEK, 316 L) thereby avoiding any potential interactions with the veins. The grafts were brought to their initial length using the adjustment device. Deaeration was performed by using two three way stop cocks. An overview showing the components of the perfusion system is given in Figure 1B. Under arterial pulsatile (Figure 1C) and non-static flow conditions three veins were cultured for one day, five veins for three days and four veins for five days. To establish the reliability of the system we perfused five HSVGs for one, three veins for three and four veins for five days with low pressure conditions (10 mmHg, flow rate 5 ml/min) which mimics the physiological venous pressure profile. Sensors on both side of the vessel chamber permanently surveyed the pressure inside the circuit (Figure 1B). In case of a pressure decrease a tiny volume of medium was injected into the circuit from an external medium reservoir mounted in a syringe pump. With this setup we were able to maintain the pressure constantly within a deviation of less than 2 mmHg during the whole experiment. The perfusion conditions were controlled by a customized software package. By using a PID control algorithm to control the syringe pump a constant pressure could be secured throughout the whole experiment. Pressure data were logged every 10 seconds and were analyzed after every trial.

Human saphenous veins support arterial perfusion conditions for one week

Under venous conditions all tested veins contained viable cells throughout the vessel wall for up to 12 days indicated by a conversion of MTT into a purple formazan product (data not shown). Thereafter, the viability dropped (Figure 2A). We then analyzed to what extent the veins would support an elevated pressure which corresponds to the arterial situation (100 mmHg, flow rate 50 ml/min). After one and four days of arterial perfusion all veins were fully viable (Figure 2B) and showed an intensive purple staining. Even after seven days the cells clearly showed metabolic activity though to a reduced degree (Figure 2B). Beyond one week the veins did not support these elevated pressure conditions evidenced by the complete lack of MTT conversion (Figure 2B). Thus, we have successfully established a standardized system, which allows the perfusion of human veins with an arterial pressure profile for up to one week.

To further explore potential pathological changes in HSVGs upon perfusion, we investigated tissue sections from veins after perfusion with venous or arterial pressure profiles at different time points by a hematoxylin/eosin staining. As a reference, we used an unperfused section of the same vein. Exposure to venous pressure for three days did not change the histology and even after five days a minor thickening of the intimal layer was evident (Additional File 1, Figure S1). After arterial perfusion for one day also no major changes could be noticed. However, after three days the intimal layer started to visibly thicken and after five days extensive hyperproliferative areas were seen (Additional File 1, Figure S1).

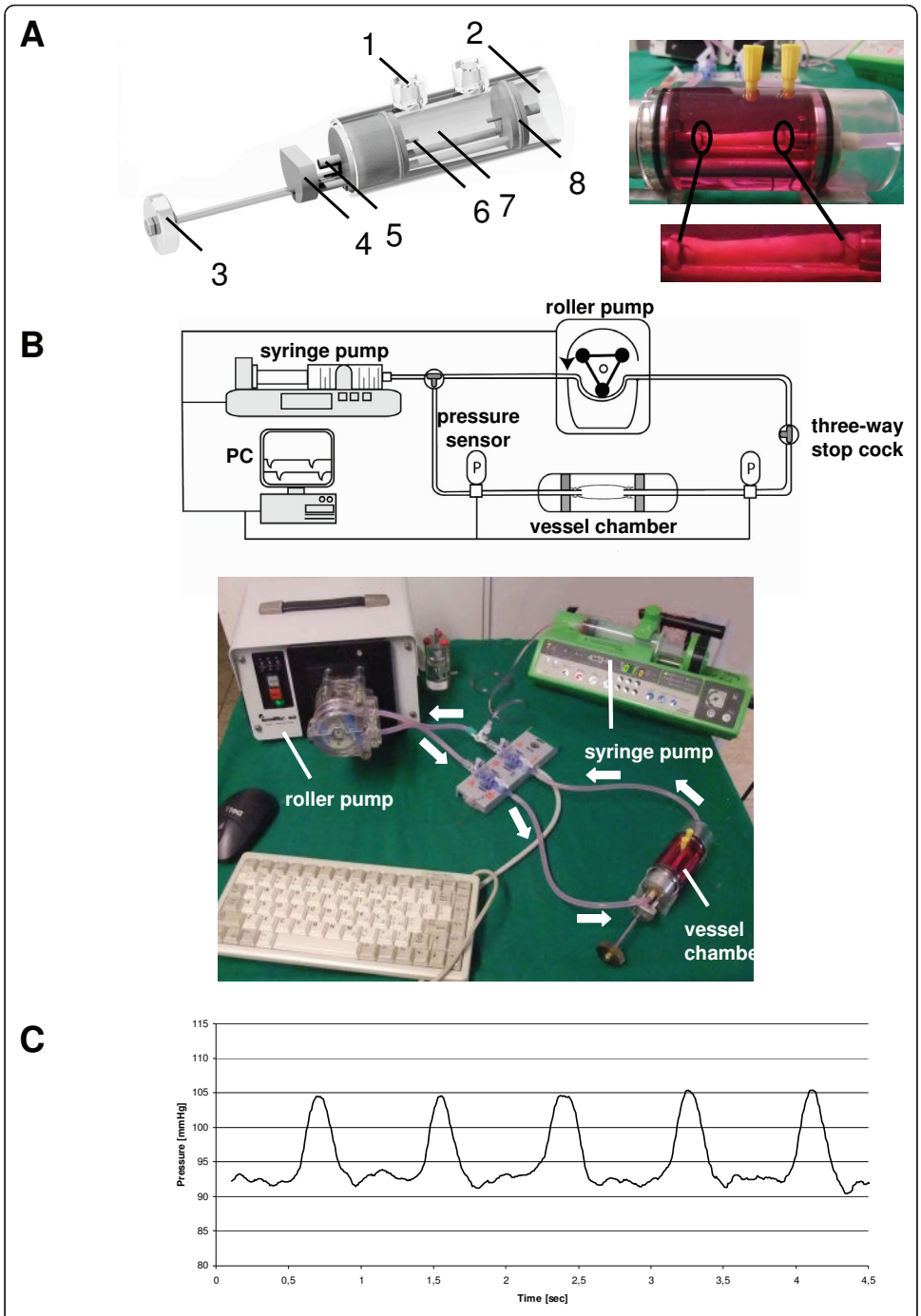

Figure 1 *Ex vivo* perfusion system. **A)** Vessel chamber. 1: ports for medium exchange, 2: glass tubing, 3: rotating wheel for length adjustment of sliding unit, 4: sliding unit, 5: Luer connector to circuit, 6: tapered adapter to fix veins, 7: vessel chamber, 8: sealing ring. **B)** Components of the perfusion system. **C)** Logged pressure time profile.

Arterial perfusion conditions up-regulate MMP-2 gene and protein expression

We next addressed the question whether the system is suitable to record alterations in gene expression as a consequence of exposure to different pressure profiles. To that end we analyzed MMP-2 as its expression is known to increase as a consequence of hypertension and vein graft preparative injury [20-22]. We first determined MMP-2

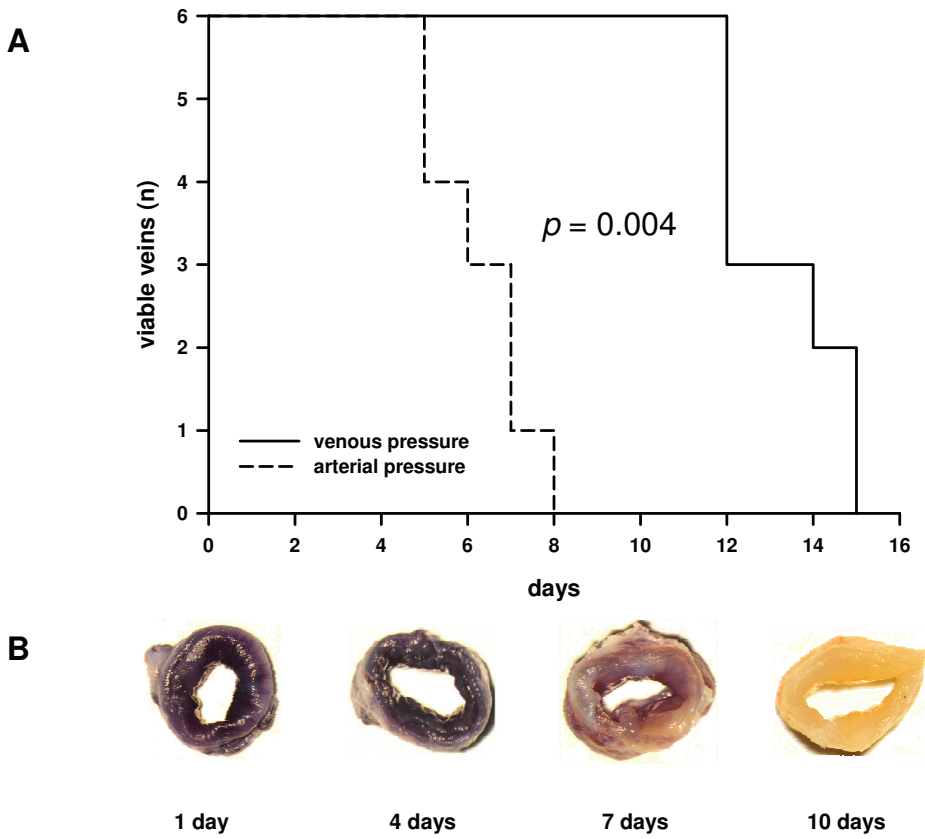

Figure 2 Viability of human saphenous vein grafts under different pressure conditions. **A)** Veins were perfused with either venous (10 mmHg) or arterial pressure profiles (100 mmHg). Viability of cells was tested using the MTT conversion assay as described in Material and Methods. *n* = 6 for both conditions. **B)** Representative MTT stainings of HSVGs at different time points.

expression in human veins which were perfused with 10 mmHg for one day which revealed a baseline ratio of MMP-2/β-actin of 2.2 ± 0.5 (n = 5) compared to unperfused control tissue (Figure 3). Extended perfusion of HSVGs for three days gave a similar result (3.7 ± 0.6, n = 3) and perfusion for five days under venous conditions showed a slightly increased gene expression of 5.0 ± 1.0 (n = 4) (Figure 3). No significant difference could be observed between venous perfusion of HSVGs for one or three days. Perfusion with 10 mmHg revealed statistical significance between five days and one day ($p < 0.05$) (data not shown), probably due to the elongated exposure in the *ex vivo* system. Perfusion of HSVGs with 100 mmHg for one day yielded an MMP-2 gene expression ratio which was similar to the reference (2.4 ± 0.4; n = 3) (Figure 3). However, MMP-2 gene expression was significantly up-regulated when HSVGs were exposed to an arterial perfusion profile for three days (19.8 ± 4.3; n = 5). This value increased further when arterial conditions were extended to five days (23.9 ± 6.1; n = 4) ($p < 0.05$; Figure 3). Thus, the elevation of MMP-2 gene expression starts rapidly when HSVGs are exposed to arterial flow conditions and it is maintained at this high level for at least five days. We then determined whether this change in RNA expression was also reflected on the protein level in a zymographic analysis. Under venous pressure MMP-2 activity corresponding to a molecular weight of 72 kD was detected, corresponding the activity of pro-MMP-2 (Figure 4A). Exposure to an arterial pressure

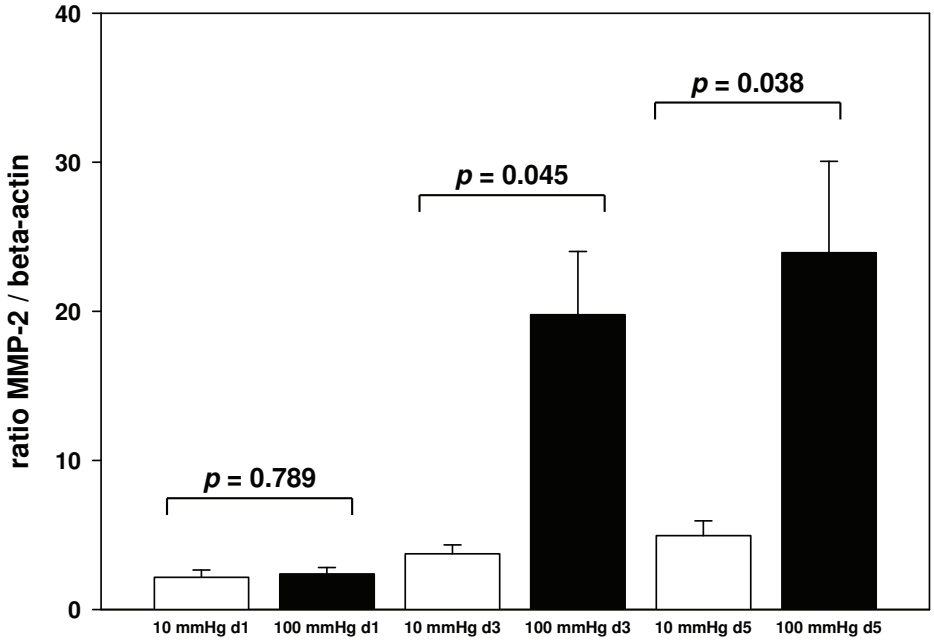

Figure 3 Increase of MMP-2 expression in human saphenous vein grafts after exposure to arterial pressure. HSVGs were perfused with venous (10 mmHg, white bar) or arterial (100 mmHg, black bars) pressure profiles for the indicated time relative to the unperfused controls of the same veins, respectively. 10 mmHg 1 day (n = 5), 10 mmHg 3 days (n = 3), 10 mmHg 5 days (n = 4), 100 mmHg 1 day (n = 3), 100 mmHg 3 days (n = 5), 100 mmHg 5 days (n = 4). The MMP-2/β-actin ratio in unperfused veins was arbitrarily set to 1.

for one day yielded similar patterns (Figure 4A). However, when arterial pressure profiles were applied for three or five days gelatinolytic activities were strongly increased. In particular, the 63 kD form of MMP-2 showed a heavily increased activity when compared to unperfused control tissues (Figure 4A). Quantification of the gelatinolytic activity confirmed our results of MMP-2 mRNA expression (Figure 4B). Gelatinase activity did not increase significantly between venous (2.1 ± 0.4, n = 5) and arterial (3.4 ± 1.4, n = 5) perfusion after one day. According to the results of mRNA expression extended perfusion with arterial pressure for three (4.5 ± 1.4, n = 5) or five days (5.6 ± 0.9, n = 5) revealed significantly elevated MMP-2 gelatinolytic activity compared to venous conditions (2.5 ± 0.6, n = 5 and 2.7 ± 0.4, n = 5, respectively).

Thus, our novel *ex vivo* perfusion system proved its ability to monitor alterations in the expression of genes which are expected to increase their activity due to elevated pressure conditions on the RNA and protein level.

Discussion

A major problem with HSVGs remains their occlusion after a certain time. Transposition of a vein segment and exposure to the arterial hemodynamic environment leads to an acute increase in flow rates and intraluminal pressure and is thought to be a potential trigger for the pathological remodeling of HSVGs [22,23]. Gene expression profiling approaches revealed that many genes and multiple pathways are differentially regulated under these conditions [24-26]. In the present study, we have established an *ex vivo* perfusion system developed to mimic the arterialization of HSVGs. The culture system provides the ability to reproduce the initial events taking place when the grafted

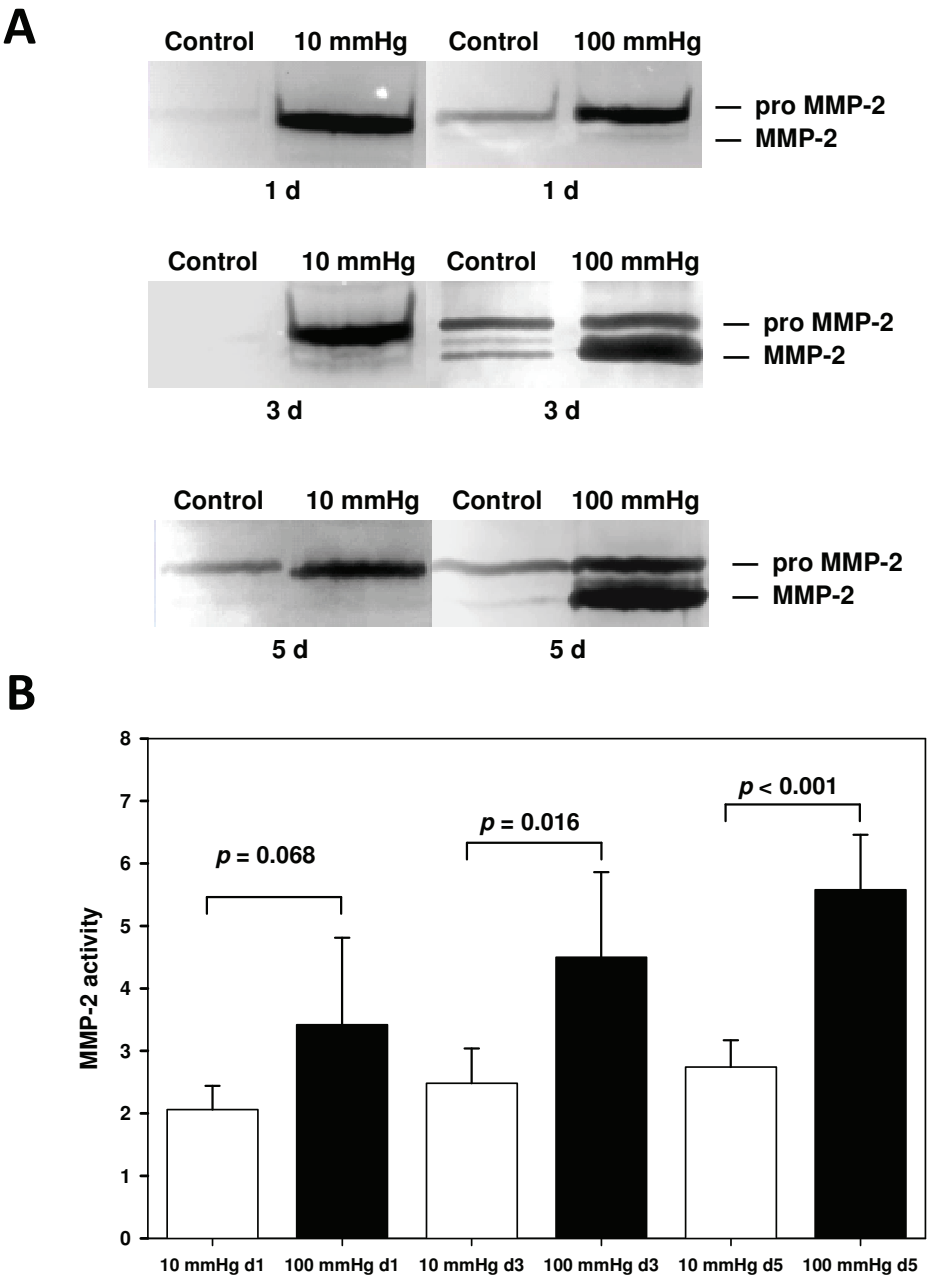

Figure 4 Zymographic analysis of MMP-2 activity. **A** Representative results are shown for HSVGs perfused with venous or arterial pressure. Controls represent MMP-2 activity of unperfused tissue taken from the same vein (n = 5 for each condition). **B** Quantitative analysis of MMP-2 gelatinolytic activity. The activity in unperfused veins was arbitrarily set to 1.

vein is exposed to arterial hemodynamic conditions. Therefore, our system may represent a valuable and reasonable approach to identify molecular mechanism underlying the early stages of bypass grafting. Several *in vitro* and *in vivo* studies have demonstrated changes in graft morphology, viability, cellular density or gene expression under arterial conditions [7,16,22,27,28]. Saucy et al. for instance used an *ex vivo* vein support system to perfuse HSVGs with arterial conditions regarding shear stress, flow rate and pressure during a period of 7 and 14 days. They found significant IH and a

marked increase in plasminogen activator inhibitor 1 (PAI-1) expression in the human veins after 7 and 14 days of perfusion [29]. A mathematical model of early vein graft IH induced by shear stress and based on experimental data with bilateral rabbit carotid vein grafts describes the general behavior of the remodeling process [23]. The group of Porter et al. demonstrated that arterial shear stress inhibits the development of IH in cultured vein pieces [30]. Previous studies have shown that SMC proliferation and migration depend on the activity of matrix-degrading enzymes. In fact, MMP-2 is an enzyme which is directly involved in vascular remodeling [14] and rodent animal models confirm that MMP-2 levels are increased under hypertensive conditions [20,21].

Within three days of perfusion under arterial pressure conditions in our perfusion system the expression of MMP-2 increased more than nine-fold and reached an even higher value after five days, similar to the activation of PAI-1 [31]. Our data are further supported by other reports which shows an increased *de novo* synthesis of MMP-2 in HSVGs perfused with artertial conditions [22] or in animal models who underwent vein grafting [8,16]. Berceli et al used a rabbit model with bilateral common carotid interposition vein grafting. They could show that accelerated IH resulting from reduction in wall shear stress was associated with an increase in MMP-2, mainly in an active form [16]. Our zymographic analyses are in accordance with their results and those of Patterson et al. [17], as we found strongly increased gelatinolytic activities in veins after perfusion with arterial pressure profiles particularly of the active form of MMP-2. As we compared HSVGs under venous or arterial pressure conditions, the elevation of MMP-2 can be attributed strongly to the arterial pressure profile. Both, gene and protein expression were significantly increased after perfusion with an arterial hemodynamic profile compared to venous conditions although all HSVGs had the same mechanical injuries after harvesting and mounting in the *ex vivo* perfusion system. Thus, the results of our perfusion system perfectly reflect the *in vivo* situation suggesting that genes which are involved in vascular remodeling are activated by arterial pressure. Therefore, our system can be used to analyze molecular parameters involved in such events in detail under standardized, tightly controlled and reproducible conditions.

An important advantage of our system is the possibility to mount vessels of variable length and diameter. The sliding unit allows a very flexible adjustment to guarantee that the vessel maintains its natural length and tension throughout the experiment. Our main focus was to setup an experimental system, which is suitable to reliably analyze molecular parameters as a function of altered pressure and flow conditions. Therefore, the most important point was to control the pressure conditions very stringently and also to keep them very stable. In pilot experiments we experienced a continuously decreasing pressure in the circuit, despite any leakage. Knowing that pressure affects gene expression such a behavior would be fatal for a desired molecular readout. With regard to this a unique feature of our perfusion system is the regulation of the mean pressure in the circuit by a computer controlled syringe pump. Decreasing pressure due to diffusion processes through out the silicone tubing [32] or relaxation of the vessel can be compensated automatically. Long time trials can be performed due to this amendment enabling an objectively constant mean pressure. In addition, up to four grafts can be perfused simultaneously within one circuit. Using MTT conversion we were able to confirm that HSVGs, which were perfused with a low-pressure profile in

our system, remained viable for up to two weeks. This is in good agreement with other reports which have estimated the integrity of the vessels by histological or immunohistochemical methods [28,31,33]. Switching the conditions to an arterial pressure profile leads to a visible reduction of the MTT staining beyond five days of perfusion. These findings are similar to those of Miyakawa et al. who detected diminished cell viability in vein segments after perfusion with arterial conditions for four days [28]. They confirmed their results by hematoxylin staining which also reveals a reduction of nuclear staining on day four [28]. Gusic et al. could even show a dramatic increase in cell death index in all layers of the graft after one week [7]. We have also performed experiments in which HSVGs were perfused with pathologically elevated pressure (200 mmHg). However, under these conditions the grafts rapidly degenerated and after two days no MTT conversion was detected any more (data not shown). Our study, like others, is limited by the inability to perfuse the *ex vivo* system with autologous blood lacking blood cells, platelets, plasma, blood-surface interaction and the multitude of inflammatory and coagulation mediators playing an important role in the pathophysiology of IH development. However, because of technical reasons, we were not able to perfuse veins with blood by using a roller pump for perfusion to achieve a pulsatile flow. Platelets would be inevitably activated and blood cells destroyed during passage through the pump. Hemolysis could be avoided or highly reduced by using a centrifugal pump instead, which in turn produces a nonpulsatile flow. Inclusion of blood would provide exposure of the vein to a more physiological state, but may also confound the results with numerous other variables. Another limitation is the time-restricted viability of grafts in the *ex vivo* perfusion system which would not be prolonged by blood perfusion due to the accumulated metabolic waste products and inflammatory reactions. Despite these limitations, the findings of the current study highlight important potential in our understanding of the healing and adaptation of veins transplanted to the arterial environment. From the beginning of the development we tried to keep the total volume of the circuit relatively small. At present it comprises approximately 20 ml which is substantially lower compared to other systems which use volumes up to 500 ml [30,34]. If necessary the circuit can be scaled down even further to a volume of approximately 10 ml. Exogenous substances can be added in a defined concentration with a reasonable and affordable consumption of material, even during long-term experiments with repeated changes of medium and substances. One conceivable scenario is the induction of an inflammatory reaction in the vein followed by the addition of recently developed anti-inflammatory drugs [35,36]. Another most obvious application is the use of small molecules which have shown their anti-angiogenic potential *in vitro* [37]. Our system might unveil novel aspects about the activity of such molecules as the affected endothelial cells are located in their natural environment and maintain their physiological interactions with other cell types.

Conclusions

In summary, we have developed a novel *ex vivo* perfusion system which maintains human veins viable for up to two weeks under a low pressure profile. The setup guarantees a tightly controlled and stable perfusion rate and the system proved to be suitable to record alterations in gene and protein expression induced by different perfusion profiles. Further advantages of our system are a total flexibility concerning the size of

potential vessels and almost infinite possibilities in various research areas by the addition of defined amounts of exogenous substances into the circuit. Our *ex vivo* perfusion system and its applications may, therefore, help to improve the long-term patency of human bypass grafts.

Additional material

Additional File 1: Figure S1. Histological analysis of a representative formalin fixed and paraffin-embedded HSVG after perfusion with different pressure profiles and hematoxylin/eosin staining. The control represents the unperfused vessel. The other parts of the vein were perfused with physiological venous (10 mmHg) or arterial pressure (100 mmHg) for the time indicated.

Acknowledgements
We gratefully acknowledge the excellent support of Christian Becker in the establishment of the controlling software. This work was supported by institutional and departmental sources from the Department of Cardiovascular Surgery of the German Heart Center. The authors gratefully acknowledge the support of the TUM's Faculty Graduate Center Weihenstephan at Technische Universität München.

Author details
[1]German Heart Center Munich at the Technische Universität München, Department of Cardiovascular Surgery, Lazarettstrasse 36, D-80636 Munich, Germany. [2]Technische Universität München, Research Center for Nutrition and Food Science, Nutritional Medicine Unit, Gregor-Mendel-Strasse 2, 85350 Freising-Weihenstephan, Germany.

Authors' contributions
- **SD** prepared the human saphenous vein grafts and mounted them into the perfusion system, tested the viability of the vein grafts, performed quantitative RT-PCR-analysis and wrote the major part of the manuscript
- **SE** constructed the set-up of the perfusion system and helped with the development of the software
- **CT** helped to prepare and mount the HSVGs, participated in the quantitative RT-PCR-analysis and performed the zymographic studies
- **US** participated in the design and helped to build the perfusion device
- **BV** harvested the HSVGs for the perfusion system and viability experiments during CABG surgery and helped to draft the manuscript
- **MAD** participated in harvesting the HSVGs for the perfusion system and viability experiments during CABG surgery and helped to draft the manuscript
- **HH** participated in the design and coordination of the study and helped to draft the manuscript
- **HL** participated in the design of the study, performed statistical analyses and participated in writing of the manuscript
- **RL** harvested the HSVGs for the perfusion system, participated in the design of the study and its coordination and helped to draft and improve the manuscript
- **MK** initiated and designed major parts of the study, participated in coordination and writing of the manuscript

All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 18 May 2011 Accepted: 21 July 2011 Published: 21 July 2011

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doi:10.1186/1475-925X-10-62
Cite this article as: Dummler *et al.*: Pulsatile *ex vivo* perfusion of human saphenous vein grafts under controlled pressure conditions increases MMP-2 expression. *BioMedical Engineering OnLine* 2011 **10**:62.

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