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# MOLECULAR MECHANISMS OF INFLAMMATION AND CALCIFICATION IN AORTIC VALVE STENOSIS

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*To my family*

## ABSTRACT

Aortic valve stenosis is a slowly progressive disorder with a spectrum of disease ranging from aortic sclerosis to severe destroyed valvular architecture leading to critical outflow obstruction. The diseased valve is characterized by inflammation, as an initiating event, pathological remodeling of extracellular matrix and pronounced calcification, which all eventually cause restricted leaflet mobility. Compelling evidence obtained from both experimental animal models and human studies provided detailed histopathological picture of disease development. This implies endothelial cell-, macrophage activation and inflammation-dependent calcification paradigm, in which phenotypic transdifferentiation of valvular myofibroblasts towards osteogenic phenotype takes place to further enhance the structural and compositional changes of the valve leaflets. The prognosis of symptomatic patients with severe stenosis is poor without surgical valve replacement. To date there is no medical treatment for this condition other than surgical valve replacement.

Based on inflammation associated calcification, observed in early stages, within this thesis the molecular mechanisms of calcified aortic valve stenosis with focus on inflammation and subsequent calcification were characterized. The aim of the project was to determine the role of proinflammatory signaling through the leukotriene pathway in aortic stenosis. Surgically explanted human aortic valves were subjected for macroscopic dissection followed by Taqman qPCR in order to correlate the gene expression pattern with the echocardiographic parameters quantifying the stenosis severity. This study established a macroscopic dissection technique as a model for *in vivo* disease development, where different parts of stenotic aortic valve represent the entire disease spectrum from early signs to advanced stages. The inflammatory environment within the affected aortic valve stimulates the 5-lipoxygenase pathway leading to production of potent inflammatory mediators, leukotrienes. Several components of the 5-lipoxygenas pathway were correlated with the stenosis severity. As inflamed valvular tissue demonstrates signs of micro- and macrocalcification, the next step was to determine the spatio-temporal distribution of genes with osteoinductive and osteoresorptive pontential. These findings along with the plasma measures of proteins taking part in bone turnover point to a correlation between their tissue-, systemic levels and the stenosis severity. Furthermore, the intracellular effects of $LTC_4$ *in vitro* cell culture of valvular insterstitial cells and smooth muscle cells from human coronary artery were characterized. The results of these experiments demonstrated cascade of events leading to activation of cell death pathways, activation of the nuclear enzyme PARP-1, upregulation of $CysLT_1$ receptor expression in response to proinflammatory stimuli coupled to nuclear calcium signaling. Moreover, in a separate experiment a conceptual model of phenotypic plasticity of the valvular insterstitial cells due to epigenetic alteration was provided, which might be connected to stenosis progression. The amount of methylated DNA within the 5-lipoxygenase promoter region was significantly lower in the calcified part compared with non-calcified, which was confirmed by accompanied increase in expression of 5-lipoxygenase leading to enhanced inflammatory activity in the calcified region of the valve.

In conclusion, the findings of this thesis integrate observations of molecular research using quantitative gene expression data with clinical variables in terms of echocardiographic parameters. The results point to leukotrienes as potential mediators of inflammation in aortic stenosis. Furthermore, the bone turnover both at tissue and systemic levels is associated with stenosis severity. In addition, the thesis also provides mechanistic insight into the direct role of leukotrienes in the pathophysiological process of aortic stenosis. Finally, translational implication of our data suggests possibility for pharmacological intervention using leukotriene receptor antagonists with a potential to retard the hemodynamic progression.

**LIST OF PUBLICATIONS**

This thesis based on the following original studies which will be referred to by their Roman numerals.

I. **Edit Nagy**, Daniel Andersson, Kenneth Caidahl, Maria J. Eriksson, Per Eriksson, Anders Franco-Cereceda, Göran K Hansson, and Magnus Bäck
Upregulation of the 5-lipoxygenase pathway in human aortic valves correlates with severity of stenosis and leads to leukotriene-induced effects on valvular myofibroblasts. *Circulation.* 2011;123:1316-25.

II. **Edit Nagy**, Kenneth Caidahl, Anders Franco-Cereceda and Magnus Bäck
Increased transcript level of poly(ADP-ribose) polymerase (PARP-1) in human tricuspid compared with bicuspid aortic valves correlates with the stenosis severity. *Biochem Biophys Res Commun.* 2012;420:671-5.

III. Alison Eaton, **Edit Nagy**, Jérémy Fauconnier, Mathilde Pacault, Magnus Bäck
Cysteinyl-leukotriene signaling through perinuclear CysLT1 receptors on vascular smooth muscle cells transduces nuclear calcium signaling and alterations of gene expression. *J Mol Med.* 2012; Apr 20. [Epub ahead of print].

IV. **Edit Nagy**, Magnus Bäck
Epigenetic regulation of 5-lipoxygenase in the phenotypic plasticity of valvular interstitial cells associated with aortic valve stenosis. *FEBS Letters.* 2012; 586,1325-1329.

V. **Edit Nagy**, Per Eriksson, Mohamed Yousri, Kenneth Caidahl, Erik Ingelsson, Göran K. Hansson, Anders Franco-Cereceda, Magnus Bäck
The role of valvular osteoclasts in calcification and aortic valve stenosis severity. (manuscript)

## CONTENTS

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| AA | Arachidonic acid |
| AS | Aortic valve stenosis |
| AV | Aortic valve |
| aVIC | activated valvular interstitial cell |
| AVA | Aortic valve area |
| AVA/BSA | Aortic valve area indexed for body surface area |
| AdC | 5-Aza-2'-deoxycytidine |
| BAV | Bicuspid aortic valve |
| BGLAP | Osteocalcin |
| BMI | Body mass index |
| BMP-2 | Bone Morphogenetic Protein-2 |
| BMP-6 | Bone Morphogenetic Protein-6 |
| BMU | Bone multicellular unit |
| cDNA | Complementary deoxyribonucleic acid |
| CD 68 | Macrophage marker |
| CD 8 | T-lymphocyte marker |
| Ct | Cycle threshold |
| CysLT | Cysteinyl-Leukotrienes |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| EC | Extracellular |
| ECM | Extracellular matrix |
| ELISA | Enzyme-linked immunosorbent assay |
| FLAP | 5-Lipoxygenase activating protein |
| Hs CRP | High sensitive CRP |
| HMG-coenzyme A | 3-hydroxy-3-methylglutaryl coenzyme A |
| IFN-γ | Interferon gamma |
| IL-6 | Interleukin -6 |
| IL-1β | Interleukin-1beta |
| LDL | Low density lipoprotein |
| 5-LO | 5-Lipoxygenase |
| LPS | Lipopolysaccharide |
| LT | Leukotrienes |
| $LTA_4H$ | Leukotriene $A_4$ hydrolase |
| LVOT | Left ventricle outflow tract |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| OPG | Osteoprotegerin |
| OPN | Osteopontin |
| PAI-2/SERPIN B2 | Plasminogen activator inhibitor-2 |
| PARP-1 | Poly-ADP-ribose polymerase 1 |
| P-max | The maximum value of transvalvular pressure gradient |
| P-mean | The mean value of transvalvular pressure gradient |
| qPCR | quantitative polymerase chain reaction |
| RANK | Receptor activator of nuclear-κB |
| RANKL | Receptor activator of nuclear-κB ligand |

| | |
|---|---|
| ROS | Reactive oxygen species |
| Runx2/Cbfa1 | Runt-related transcription factor |
| SMC | Smooth muscle cell |
| TAV | Tricuspid aortic valve |
| TNF-α | Tumor necrosis factor alpha |
| TGF-β | Transforming growth factor beta |
| TRAP | Tartrate-resistant acid phosphatase |
| VIC | Valvular insterstitial cell |
| V-max | The maximum velocity across the aortic valve |
| VTI-ratio | Velocity time integral ratio |
| Wnt | Wingless/Int |

## PREFACE

Calcified aortic valve stenosis is increasing in prevalence and has become the most common indication for surgical valve replacement.
Aortic valve stenosis is a slowly progressive disorder with a disease continuum beginning with early phases, called aortic valve sclerosis resembling to early atherosclerotic plaque formation, however which may subsequently progress toward to more severe calcification and ultimately to a critical stenosis.
In addition, aortic valve stenosis is characterized by inflammation, a key feature preceding pronounced calcification of the leaflets, leading to pathological remodeling of the valvular architecture. To date, there is no medical treatment of the aortic valve stenosis other than surgical valve replacement. Therefore it is emerging to pursue new pathways, which could be a potential target of treatment in a force to achieve hemodynamic retardation.

This thesis is focusing on some of the key features of the aortic valve stenosis including inflammation and calcification, through demonstration of the involvement of the leukotriene pathway with its functional consequences. Moreover, to further explain the potentially deleterious effects of leukotrienes in the valvular tissue and on valve's structural cells, demonstration of several mechanistic experiments are also included in the thesis.
Finally, an overview of the current understanding of the aortic valve stenosis development and progression will be discussed including the importance of our studies in this context.

# 1 INTRODUCTION

## 1.1 THE NORMAL AORTIC VALVE

### *1.1.1 The anatomy and physiology of aortic valve (AV)*

A competent aortic valve (AV) with three underlying semilunar cusps ensures unidirectional blood flow throughout the cardiac cycle with minimal obstruction and without regurgitation[1]. Multiscale computational simulations linking cell-, tissue-, and organ-scale models demonstrated flow convergence and unobstructed forward flow under physiological conditions with underlying TAV[2]. The AV has a tissue structure with only a sparse vasculature[3] at its proximal part and its motions are driven by mechanical/hemodynamic forces exerted by the surrounding blood. The ability of the valves to permit unobstructed forward flow depends on the mobility, pliability, and structural integrity of their cusps[1]. Schoen has provided a detailed description about the anatomy of the AV[1], a complex system maintaining the adequate stroke volume and cardiac output, adjusted to the instantaneous functional requirements of the circulation. The individual AV cusps attach to the aortic wall in a crescentic semilunar fashion, ascending to the commissures and descending to the basal attachment of each cusp to the aortic wall. The aortic root behind the cusps are dilated, this area called *sinuses of Valsalva*, which stretch out with each ejection of blood. The three separate AV cusps with their respective equal sized sinuses have a relationship to the coronary artery ostia that they arise from them, normally a left, a right, and a noncoronary cusp. Coaptation of the free parts of the cusps ensures complete central closure of the valve during diastole[1]. Due to physiological changes of the transvalvular pressure gradients during the cardiac cycle, different parts of the AV are exposed to mechanical forces of the bloodstream. For example, while under systole the AV's inflow surface containing the *ventricularis* layer is exposed to pulsatile and oscillatory shear stress, relevant changes in hemodynamic forces begin when the bloodstream decelerates in the aorta at the end of systole causing vortices in the sinuses and facilitating valve closure[1]. The diastolic coaptation of the AV depends on the relation/pressure gradient between "the bending stretch" occurring in the outflow surface right beneath the semilunar cusps and "the tensile stretch" in the outflow surface right above the semilunar cusps[4]. Under systole the cups are relaxed due to their elastin content allowing rapid, reversible deformation and prompt accommodation, while under diastole the valvular tissue is stretched and occludes the orifice. The proper function of a competent AV depends on the integrity and the highly organized/coordinated movements of the cuspal attachments[1].

The AV consists of three principal layers beneath the valvular endothelial cells that envelope both the inflow and outflow surfaces of the valve. The *spongiosa* layer made up of loosely organized connective tissue localized on the ventricular side, closed to the outflow surface rich in glycosaminoglycans and scattered valvular interstitial cells. The *fibrosa* layer is the most superficial layer of a cusp located beneath the valvular endothelial cell layer on the aortic side and is distinguished by its richness of densely packed collagen. The *ventricularis* layer contains mainly elastin fibers perpendicularly orientated from the base to the free edge of the cusp. The maintenance of the valve integrity and its function is closely connected to the aortic valves own structure cell, i.e. the valvular interstitial cells, a mesenchymal cell type with many similarities to the smooth muscle cells and fibroblasts, however representing a heterogeneous cell population with high phenotypic plasticity. Beyond the VICs, valvular endothelial cells, high concentration of organized collagen, elastin fibers and glycosaminoglycans build up the valvular ECM. The components of the valvular ECM, mainly collagen fibers demonstrate cyclical internal rearrangement, in terms of progressive rotational alignment and extension of fibers in response to pulsatile and oscillatory changes of transvalvular pressure gradients[1], that occur under normal valvular function. Because of strains under diastole, when the valve is closed and the mechanical properties of the AV's cusps are anisotropic, the compliance exhibits differences demonstrating more competence in radial than in circumferential direction. This radial compliance is closely connected to the *ventricularis* layer, rich in elastin fibers[5].

### *1.1.2 Aortic valve interstitial cell (VIC)*

Valvular interstitial cells (VICs), the dominating cell type of aortic valves, are responsible for the maintenance of valve integrity. The interstitial cells act as mechanical sensors through complex cell-ECM interactions and translate forces to the cellular level as mechanotransductors, which is critical in the maintenance of the valve integrity and its architecture. VICs represent a heterogeneous cell population and demonstrate phenotypic plasticity, which is crucial for valve development during organ genesis and even involved into the valvular disease development and progression. Phenotypic transdifferentiation of VICs, determined as an altered expression profile of cytoskeletal and surface proteins, reflects an age-related adaptation mechanism of the valvular structure, which is associated with a physiological maturation process[6]. VICs hence represent a highly plastic cell population with dynamic phenotypic features, including different subpopulations that may exhibit embryonic progenitor endothelial/mesenchymal cells (eVICs), quiescent VICs (qVICs), activated VICs (aVICs), postdevelopmental/adult progenitor VICs (pVICs), and osteoblastic VICs (obVICs)[7].

Under pathological conditions, a subset of VICs may undergo phenotypic transdifferentiation toward bone-producing osteoblast-like cells with expression of osteogenic mediators, such as osteocalcin, bone sialoprotein, and osteonectin, which participate in the active calcification associated with aortic stenosis[8, 9].

The embryonic progenitor endothelial/mesenchymal cells undergo endothelial-to-mesenchymal transformation (EMT), which is connected to the valve formation in the fetus. Whithin the valvular morphogenesis, there is a complex interplay amongst myocardial and endocardial signaling which is necessary for EMT. The endocardium starts to secrete TGF-β, and together with BMP-2 secreted by the myocardium, acts synergistically to enhance mesenchyme formation[10].

The qVIC are the cells that are at rest in adult and responsible for the maintenance for the normal valve physiology.

The aVICs, expressing α-actin, as an evidence for their activation to become myofibroblasts regulate the pathobiological responses of the valve. aVICs are the master cells in diseased valves and exhibit heterogeneity in motility. The activated state of this versatile subpopulation of VICs (aVICs) is associated with increased synthetic activity in terms of increased ECM secretion, expression of MMPs, and increased proliferating potential and migration properties. This pathologically increased synthetic activity contributes to compositional changes and altered quantity of the ECM upon cell activation. Moreover, aVICs release a number of cytokines, one of the most important is TGF-β, which acts in autocrine manner.

The pVICs consists of heterogeneous population that may take place in valve repair.

The obVIC the best characterized of all the above mentioned subtype and regulate chondrogenesis and osteogenesis in situ[7]. Earlier findings observed in human aortic valve lesions provided evidence that atherogenic risk factors including oxidized LDL, upregulate the expression of BMP-2 leading to promotion of osteogenic signaling pathways, such as Runx2/Cbfa1[11,12] and Wnt/Lrp5/β-catenin[13] leading to osteoblastic differentiation with subsequent calcification. The Lrp 5 (LDL receptor related protein) belongs to the LDL-receptor family and mediates the intracellular effects of the canonical Wnt pathway resulting in nuclear translocation of β-catenin, which mediates the transcription of several target genes, for example Runx2/Cbfa1[14].

### *1.1.3 Vascular smooth muscle cells (vascular SMC)*

Vascular SMCs is a mesenchymal cell type sharing many phenotypic similarities with VICs. Atherosclerosis is characterized by migration of vascular SMCs from tunica media into the intima layer of the arterial wall, where they start proliferate, release ECM proteins, contribute to vessel wall inflammation together with infiltrating immune cells[15]and lipoprotein retention leading to vascular lumen obstruction[16,17]. In early atherosclerosis vascular SMCs contribute to atheroma formation through the production of proinflammatory mediators and synthesis of matrix proteins required for retention of lipoproteins[17]. This phenotypic shift of the vascular

SMCs may be due to the inflammatory environment leading to altered vascular sensitivity[18]. In addition, earlier observations on primary isolated rat aortic SMCs demonstrated phenotypic shift into a synthetic state with enhanced proliferative response evoked by LT treatment[19, 20]. Furthermore, increased vascular responsiveness to LTs was observed in human atherosclerotic coronary arteries, which demonstrated contractile response to CysLTs[21,22], whereas healthy vessels did not showed any vasoconstrictor responses.

### *1.1.4 The development of the aortic valve*

Quantitative histopathological studies demonstrated that evolution of valvular tissue architecture, cell phenotypes, proliferation rate, apoptosis continue throughout the fetal and postnatal development showing adaption to altered environmental conditions[1]. VIC density, proliferation, and apoptosis were significantly higher in fetal than adult valves. Phenotypically changed VICs, determined by altered expression of cytoskeletal and surface proteins and by proteolytic enzymes, regulate age-associated structural remodeling and adaptation of the ECM[6]. During valvulogenesis the aortic valve exhibits compositional changes and develops from bilaminal structure with sparse, loosed unorganized collagen to a trilaminar structure containing elastin in the *ventricularis* and increased collagen in the *fibrosa*. Those changes became apparent by 36 weeks of gestation but remained incomplete versus the normal adult valve structure[23]. Moreover, progressive decrease of VICs density under second to third trimester and further decrease expected throughout life. In addition, aVIC undergo evolution to a more quiescent phenotype during postnatal life. Collectively, fetal valves possess a dynamic/adaptive structure and contain cells with an activated/immature phenotype. During postnatal life, activated cells gradually become quiescent, whereas collagen matures through increased fiber thickness and alignment, which suggests a progressive, environmentally mediated adaptation[6].
Rather than viewing each signaling molecule as a separate entity, several signaling pathways (such as VEGF, NFATc1, Notch, Wnt/β-catenin, BMP/TGF-β, erbB, and NF1) integrated into physiologic steps that occur during the process of valvulogenesis, build up a network model, in there they interact with each other[24].

## 1.2 AORTIC VALVE STENOSIS

Aortic valve stenosis is the most frequent primary valvulopathy in the western world and is a consequence of either a congenital malformation demonstrating abnormal commissural attachments and fusions or a result of “degenerative” calcification. The prevalence of rheumatic aortic stenosis is however low in the industrialized countries. The condition is defined by a limited opening area of the AV, causing a fixed obstruction in the left ventricle outflow due to restricted leaflet motion, rigidity and extensive calcification[25].

### *1.2.1 Epidemiology of aortic valve stenosis*

The prevalence of aortic valve sclerosis, a prestadium of manifest valvular stenosis is reported >25% in a general population with an age > 65 years and is associated with a 50% increased risk for cardiovascular events[26]. The prevalence of manifest valvular stenosis is approximately 2,5% in a population aged >75 years[27, 28]. However, end stage calcified AS can be a result of underlying bicuspid valve anatomy as well, as a congenital malformation with a prevalence of 0.9-1.37 %[29]. Patients with bicuspid aortic valve anatomy have a faster disease progression and require valvular replacement one or two decades earlier than their counterparts with normal valvular anatomy suggesting different underlying pathology.
Although, there is an overlap between the risk factors causing atherosclerosis and aortic valve stenosis, only approximately 40-50% of patients develop overt atherosclerosis with concomitant aortic valve calcification, indicating substantial differences of these two diseases entities[30]. In Europe, the latest rapport from the Euro Heart Survey in 2003 identified 33% of all patients with AS with coexisting obstructive coronary artery disease who were subjected

for concomitant coronary artery bypass grafting surgery at the time of aortic valve replacement[31].

### *1.2.2 Risk factors coupled to development of aortic valve stenosis*

One of the largest epidemiological trial[27]comprising 5201 patients investigating the traditional risk factors for atherosclerosis to the prevalence for AV sclerosis and stenosis identified age (twofold increased risk for each 10-year increase in age), male gender (twofold excess risk), present smoking (35% increase in risk) and a history of hypertension (20% increase in risk). Other significant factors included height, high lipoprotein(a) and low density lipoprotein cholesterol levels, as independent clinical factors, which were associated with degenerative AV disease. Beyond the classical risk factors, other additional risk factors have been identified to be connected to aortic valve stenosis, such as an association with the metabolic syndrome[32] with faster hemodynamic progression rate[33] in both genders. Another important risk factor significantly influencing the prevalence of AV calcification is renal dysfunction, demonstrated both in animal models[34] and in human observational studies[25, 35-37]. In one study, elevated serum creatinine levels were correlated to increased progression rate of AS[38]. Interestingly, earlier observations suggested other etiologies to aortic valve stenosis, such as infection from the respiratory tract by Chlamydophila pneumonia, long before the need of valve replacement surgery, indicating a role of a persistent infection in the pathogenesis of AS[39]. In addition, the disturbed trace elements balance detected in the valve tissue and in serum from humans further supported the active influence of an ongoing immune process coupled to an infection[40].

### *1.2.3 Clinical manifestations and prognosis of aortic valve stenosis*

Symptomatic severe aortic valve stenosis is classified by the onset of exertional chest pain, shortness of breath, dizziness and is connected with an average survival after onset of symptoms less than 2 to 3 years[41]. More severe symptoms of aortic stenosis such as heart failure and syncope are late manifestations of the disease and identify a high-risk population. Patients with depressed left ventricle function without any other explanation for it represent a subgroup which has a clear benefit from valve replacement surgery as a class I C indication, because of improvement of long-term outcome[42] postoperatively[43] despite of high expected operative mortality rate. In the latter subgroup the perioperative risk in patients with contractile reserve, detected on dobutamin stress echocardiography was 5 % and in a subset of patients with depressed left ventricle function without contractile reserve was 32%[44].
According to previous epidemiological studies once patients with aortic valve stenosis become symptomatic will experience high event rate and rapid deterioration of their functional status, therefore those patients should be considered for elective valvular replacement surgery[45]. In the clinical decision making the first step is the risk stratification of an individual patient which includes careful physical examination. This is followed by echocardiography, a standard diagnostic tool in the evaluation of the stenosis severity. In addition, echocardiography confirms the presence of valvular calcification assesses the left ventricle geometry, function, the severity of stenosis, the extent of valvular calcification and provides prognostic information[43].
However, the management of asymptomatic patients with aortic valve stenosis is challenging. According to Otto et al., in the presence of mild obstruction further hemodynamic progression is inevitably with an average annual hemodynamic progression rate of 7 mmHg in mean transvalvular pressure gradient, an increase of 0.3 m/s in peak velocity, and a decrease of 0.1 $cm^2$/year in aortic valve area is expected[46]. Peak aortic jet velocity, which is a marker of disease severity, has been shown to be an important predictor of outcome with incrementally higher event rates among asymptomatic patients with mild, moderate, and severe aortic stenosis[45, 47, 48].
Earlier observational data described that patients with aortic valve stenosis and concomitant obstructive coronary artery disease have a poorer chance for event-free survival and a more rapid hemodynamic progression[47, 48]. Recently published data described the natural course of very severe aortic valve stenosis, defined by a peak aortic jet velocity ≥5m/s, showing that

aortic jet velocities permit risk stratification beyond the conventional definition of severe aortic valve stenosis[45].
Collectively, patients with very severe but asymptomatic aortic valve stenosis should be considered for an elective valve replacement surgery because of increased risk for rapid deterioration, and high event rate[45].

### *1.2.4 Diagnosis of aortic valve stenosis-the role of echocardiography*

In the clinical practice echocardiographic assessment is the key diagnostic tool in the diagnosis of AS. It provides accurate quantification of disease severity, assess the anatomy of the valve, the presence and extent of calcification, the mobility of the cusps, using Doppler measurements the peak velocity (Vmax) across the valve, the maximum (P-max) –and mean (P-mean) transvalvular pressure gradients, and allows calculation of the effective orifice area (AVA=aortic valve area) using the standard continuity equation. This is based on modified Bernoulli equation. Furthermore, continuity equation [AVA= (cross sectional area in LVOT x $VTI_{LVOT}$)/$VTI_{AV}$] based AVA is reliable parameter for prediction of the clinical outcome and for clinical decisions making[43]. Previous evidence supports the concept that the effective not the anatomical orifice area is a primary predictor of outcome[43]. In adults with normal resting left ventricle function the calculation of the AVA is correct, whereas in the presence of left ventricle dysfunction with low flow conditions, the valve area calculation is challenging. In a subset of patients with documented depressed left ventricle function Dobutamin stress test should be performed to differentiate between severe and pseudo-severe AS[44]. The definition of severe AS according to the ESC guidelines in the presence of normal resting left ventricle function is the following: AVA< 1$cm^2$, AVA/BSA<0.6 $cm^2/m^2$, P-mean>50 mmHg, Vmax>4 m/s, VTI-ratio <0.25[43]. These Level I recommended parameters together with clinical data and the patient's functional status are adequate in the clinical decision-making[43]. The hemodynamic changes in patients with AS include an increase in afterload, progressive left ventricle hypertrophy as a consequence of pressure overload due to stepwise progressive valve obstruction, demonstrating a maladaptive changes in left ventricle. In addition, as AS and the subsequent left ventricular hypertrophy continue to progress, the left ventricle becomes less compliant due to progressive fibrosis and this leads to elevation of the left ventricle end-diastolic pressure with symptom onset in an individual patient[49].The presence of co-existing hypertension, however confounds the accurate assessment of the stenosis severity due to imposing a double-load on the left ventricle which consists of an increased systemic vascular resistance in addition to the valvular obstruction. The contribution of this superimposed vascular component increases the global left ventricle hemodynamic load in those clinical situations and the stenosis severity may be underestimated[50]. Collectively, these echocardiographic parameters identify a subpopulation within patients with AS, which characterized by low-flow, low-gradient despite preserved left ventricle ejection fraction.

### *1.2.5 Bicuspid aortic valve (BAV) and genetics of aortic valve disease*

The bicuspid aortic valve (BAV) is a common congenital cardiac malformation with a prevalence of 0.9-1.37% and with a documented male predominance 2:1[29], which could be associated with other left-sided lesions, such as aneurysm/dilatation of the ascending part of aorta[29, 51], hypoplastic aortic arch[52], coarctation of aorta[52], sub-or supravalvular membrane in LVOT[53], and in extreme cases can be a part of multiple left-sided obstructive lesions, i.e. the Shone syndrome[54] or causes through severe obstruction of LVOT hypoplastic left heart syndrome[24]. Patients with BAV develop hemodynamically significant aortic valve stenosis one or two decades earlier than those with tricuspid aortic valve anatomy[55]. The main reason for this accelerated calcification process is thought to be a consequence of altered systolic flow distribution due to abnormal commissural attachments, fusions between the valve cusps[56]. In addition, a subset of BAV-patients with anteroposteriorly located cusps exhibiting eccentric systolic flow pattern are more prone to rapid progression. Morphologically, the prevalence of type 1 (fusion of the right and left coronary cusps) is present in about 80% of affected individuals, compared to type 2 demonstrating fusion between the right and the non-

coronary cusps in about 20% in several retrospective cohorts of surgically explanted human BAV[57, 58]. Since BAV might be associated with a number of associated conditions (as stated above), this further suggests that BAV is accompanied with dysfunctional tissue structure due to a genetic difference, as stated above. The discovery of other candidate genes, such as the endothelial nitric oxide synthase (eNOS) influencing the aortic valve anatomy, provides further evidence of this gene's functional importance through its involvement into the embryonic development[59].

The Notch1 gene (with a disease locus mapped to chromosome 9p34) encods a single-pass transmembrane receptor (2556 amino acids) whose function is a highly conserved intracellular signaling pathway involved in cell differentiation and cell fate[60]. Upon ligand binding, Notch1 receptors undergo proteolytic cleveage resulting in release of their intracellular domain. Overexpression of Notch1, a transcriptional a repressor of the Runx2/Cbfa1 pathway, which a central regulator of osteoblastic transformation and may lead to impaired osteoblastogenesis[29]. According to compelling evidence obtained in a mouse model where the Notch1 mRNA transcripts were abundant in the outflow tract mesenchyme, which gives rise to the valves and in the endocardium when septation of the common trunk occurs. In addition, Notch1 was expressed at high levels in the endothelial layer and in mesenchyme of aortic valve cusps[61], suggesting its function in valvulogenesis. However, earlier observations provided evidence that Notch1 may directly affect calcium deposition into the AV. Expression of osteoblast-specific genes, such as osteocalcin, osteopontin and other osteoblast-related genes is regulated by upstream *cis* elements specifically binding to Runx2/Cbfa1[62]. Although Runx2/Cbfa1 can be inhibited by epigenetic modifications such as by histone deacetylase activity (HDAC)[63], the evidence for Notch1-mediated Runx2/Cbfa1 inhibition was present after using trichostatin A, a potent HDAC inhibitor. In addition, a downstream effector system coupled to Notch1 was identified, which directly activates the hairy family of transcriptional repressors (Hrt1 and Hrt2), and are co-expressed in the endothelial lining of the murine aortic valve leaflets, as well as in the endocardium and in the vascular endothelium[61]. The discovery of Notch1 mutations as a cause of the spectrum of AV disease has increased the current understanding of these abnormalities within the congenital cardiology[64].

### *1.2.6 The pathophysiology of calcified aortic valve stenosis*

Based on dyslipidemic experimental models[13, 65, 66] the pathogenetic role of hyperlipidemia was implemented in the human aortic valvular disease development and was the target of treatment in large interventional trials. Other interesting finding was the detection of Angiotensin-converting enzyme (ACE) in a subset of lesion macrophages, exhibiting primarily an extracellular distribution, where they colocalized with apolipoprotein B[67]. Numerous histological studies demonstrated structural changes including inflammation[68], which is thought to be the initiating step in pathological remodeling of the aortic valve tissue, followed by ECM remodeling[69] leading to increased fibrosis and valve thickening[68] with subsequent phenotypic transdifferentiation of the aVICs towards osteoblastic phenotype[8]. At advanced stages pronounced calcification containing lamellar bone formation with microfractures, apoptotic cores[70] and neovascularisation[71] takes place to further leading to progressive compositional changes of the valvular tissue. Although, there is an overlap in clinical risk factors, as mentioned above, there are major differences between atherosclerosis and aortic valve stenosis regarding the biological direction of the disease, tissue characteristics and associated clinical event as well. The underlying mechanism to clinical event in patients with aortic valve stenosis is increased leaflet thickening and stiffness, not plaque rupture[30].

In the following paragraphs an overview will be presented about the pathophysiology coupled to stenosis development and progression focusing on the role of inflammation, the presence of oxidative stress, followed by a detailed description about the calcification process.

### *1.2.7 Inflammation*

Inflammation is a key step promoting the pathological process with superimposed calcification in aortic stenosis[8, 72]. Early lesion of aortic valve stenosis is characterized by 1) subendothelial thickening on the aortic side of the leaflet, between the basement membrane and elastic lamina; 2) large amount of extra-and intracellular lipids; 3) fine stippled mineralization; 4) disruption of the basement membrane overlying the lesion, and 5) the presence of infiltrating immune cells, such as macrophages, T cells and mast cells[68, 73, 74]. The cellular composition of human atherosclerotic plaque containing macrophages and activated T cells[75] resembles to that observed in early lesions of inflamed valvular tissue. Previous histological observations of human normal valves demonstrated scattered macrophages in the *fibrosa* and *ventricularis* layers, occasional α-actin positive cells mostly in the proximal portion of *ventricularis* but no T cells were detected[68].
In contrast, infiltrating immune cells containing macrophages, T cells[76], predominantly clonally expanded T cells (mainly highly expanded CD 8 in lineage)[77] with considerable evidence of activation, were detected in stenotic valves. This study by Wu et al. elegantly showed that a subset of limited numbers of expanded T cell clones detected in the aortic valve tissue was also identified in the blood. However, the composition of the T cell repertoire in AS differs from that seen in stable vascular lesions, where the lymphocytes are mainly polyclonal[78, 79]. Moreover, highly significant predominance of CD 8 subset among valves with bicuspid morphology was detected[77].
Molecular imaging tools to quantify and visualize the components of inflammation have been used in atherosclerosis research demonstrating macrophage infiltration directly connected to local calcification[80]. Based on this knowledge, and on results of studies[81-84] showing the role of macrophage-derived inflammatory cytokines (IL-1β, TNF-α, IL-6, TGF-β) in promoting osteogenic differentiation of vascular smooth muscle cells, *in vivo* molecular imaging technique was implemented in the assessment of AS[80] and this allowed to obtain a detailed picture of the pathological process at molecular level. The introduction of the near- infrared fluorescence imaging to map endothelial cells, macrophages, proteolytic activitiy, aVICs and osteogenesis has led to paradigm shift through demonstration a concept of inflammation-dependent calcification. This process can be divided into three phases, such as initiation, propagation and end-stage ossification[80]. In the initiation phase activated macrophages infiltrate the valvular tissue and release proinflammatory and pro-osteogenic cytokines (as stated above)[85]. In the propagation phase further compositional changes take place due to the presence of excessive levels of proteolytic enzyme activity in form of MMPs and cysteine endoproteases, released by the activated macrophages, mast cells and aVICs resulting in disruption of collagen and elastin fibers, which together with matrix vesicles and apoptotic bodies may provide a core for *in situ* calcification. And finally, the end-stage characterized by heterotopic bone formation, where the intact well-functioning aortic valve is replaced by pronounced calcification containing elements of lamellar bone with microfactures[70], apoptotic cores[86-88] with preceding endochondrial bone tissue[70, 89] resembling to that existing in skeletal bone which is the key feature in advanced stages of the stenotic lesions with subsequent hemodynamic consequences.

#### *1.2.7.1 The Leukotriene pathway*

Within the inflammatory pathway, the downstream components of the arachidonic acid (AA), known as the leukotrienes (LT) induce inflammatory signaling through activation of specific BLT and CysLT receptors. The activation of the cytosolic form of phospholipase A2, exhibits higher specificity for phospholipids[90] containing AA. This step deliberates the AA followed by enzymatic lipoxygenation by the enzyme of 5-lipoxygenase (5-LO). This enzymatic step is assisted by the FLAP (5-LO activating protein), which stabilizes 5-LO and enhances its interaction to the substrate leading to production of an unstable metabolite: the $LTA_4$. The latter product can either be metabolized by the action of $LTA_4$ hydrolase yielding to production of $LTB_4$, a potent chemoattractant for neutrophils and T cells, or can be conjugated with gluthation assisted by $LTC_4$ synthase leading to production of $LTC_4$. Subsequently, the released $LTC_4$ undergoes conversion into $LTD_4$ and $LTE_4$, collectively

referred to as cysteinyl-LTs[91] (Figure 1). Alternatively, non-myeloid cells as acceptor cells expressing distal $LTA_4$-metabolizing enzymes, such as $LTC_4$ synthase can take up $LTA_4$ from a donor cell, (for example a neutrophil) and metabolize it to the final product of $LTC_4$ which mediates sustained broncho-and vasoconstriction[92]. This process is defined as transcellular biosyhthesis and exists in vascular smooth muscle cells, endothelial cells and in platelets[93].

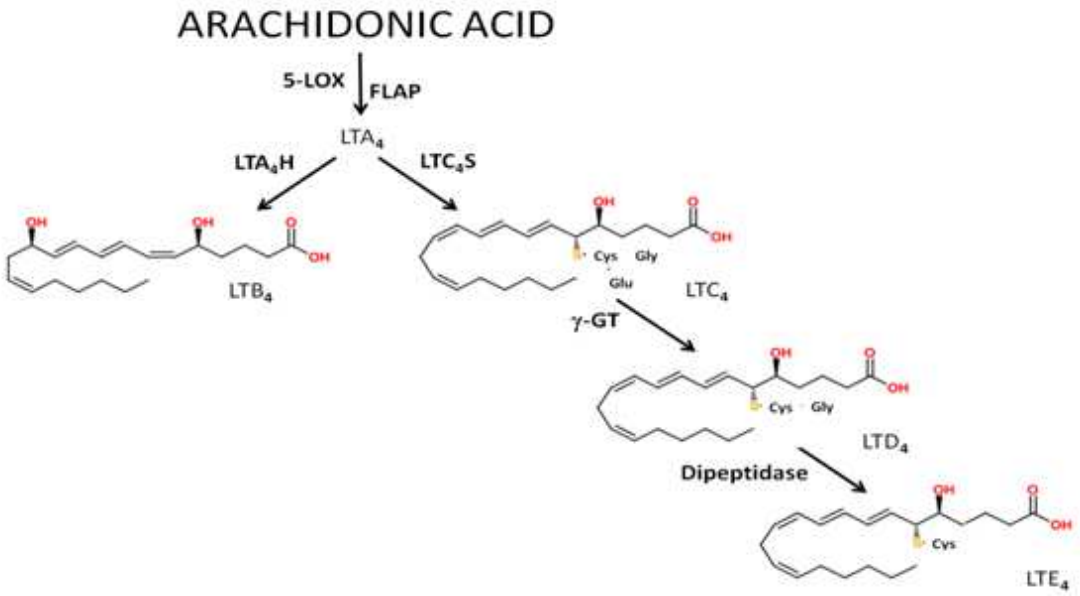



*Figure 1. The Leukotriene pathway, modified from [94].*

$LTB_4$ is released by monocytes, macrophages and neutrophils, whereas cysteinyl-LTs are mainly formed by macrophages, mast cells and eosinophils[95]. LTs act via specific receptors, divided into two subtypes, namely the BLT and the CysLT receptors, respectively and a subdivision into BLT(1) and BLT(2) receptors and CysLT(1) and CysLT(2) receptors has been established, belonging to the G-protein coupled receptors[94, 96]. Within the BLT receptor subtype, $BLT_1$ is a high affinity, whereas $BLT_2$ is a low affinity receptor of $LTB_4$. The cysteinyl-LTs ($LTC_4$, $LTD_4$, and $LTE_4$) exert their action through activation of specific $CysLT_1$ and $CysLT_2$ receptors. $CysLT_1$ activated by the CysLT with a rank order potency of $LTD_4$>$LTC_4$>$LTE_4$, whereas $CysLT_2$ agonist rank order potency is $LTD_4$=$LTC_4$, while $LTE_4$ is less potent[94]. Selective $CysLT_1$ receptor antagonists are used in the treatment of bronchial asthma. The role of the $CysLT_2$ receptor subtype has been implicated in the context of inflammation, vascular permeability and tissue fibrosis[91].

The role of LTs in the pathogenesis of cardiovascular disease, with focus on atherosclerosis has been the subject of intense investigation both in experimental settings and in human conditions.

Observational studies provided evidence for the presence of 5-LO in human atherosclerotic aorta, diseased coronary- and carotid arteries[97]. These notions were further expanded by mechanistic experiments demonstrated functional implications of the LT pathway at several stages of the atherosclerotic process[34].

Moreover, genetic variations with a decreased number of CpG-repeats in the 5-LO promoter have been reported to have a reduced susceptibility to methylation[98]. These polymorphisms identified a subpopulation with increased measures of subclinical atherosclerosis[99], hence supporting the notion of an important epigenetic regulation of 5-LO activity in cardiovascular pathology. Genetic variants of other components of the LT-pathway such as FLAP and $LTA_4$ hydrolase showed associations with cardiovascular disease and atherosclerotic complication with subsequent myocardial infarction and stroke[100-102].

In addition, a recent observation on vascular smooth muscle cells from human atherosclerotic lesion showed up-regulation of $BLT_1$ receptor mediating proliferation and migration of coronary artery SMCs, suggesting the pathogenetic role of $BLT_1$ receptor activation in response to $LTB_4$ in intimal hyperplasia[103]. $LTB_4$ is thought to promote plaque vulnerability

by increasing the activity of MMPs[104], which was further confirmed in the study evaluating the functional involvement of the LT-pathway in human abdominal aortic aneurysm[105].
Earlier investigations suggested different CysLT receptorial distribution in arterial wall in resting and in inflammatory conditions, respectively[22], demonstrating significantly upregulated transcript levels of the $CysLT_1$ receptor (three-fold) in human carotid atherosclerotic lesion[106] compared to $CysLT_2$ expression. Further studies confirmed a potential causal relationship between the high concentrations of CysLTs in gingival crevicular fluid and increased atherosclerosis burden by showing increased carotid artery wall thickness, regardless of the dental status[107].
The role as effective vasoconstrictors of cysteinyl-LTs in diseased human coronary arteries was described by Allen et al.[22], suggesting increased vascular sensitivity to LTs during atherogenesis[92]. In addition, the notion of cysteinyl-LTs as potential effectors of atherosclerosis has also confirmed by animal models showing beneficial effects on atherosclerosis burden[108,109] and intimal hyperplasia[110] by using specific antagonists of the $CysLT_1$ receptor. Collectively, these findings suggest a major role of the cysteinyl-LTs in the pathogensis of atherosclerosis. In addition, a recently published Swedish nationwide population-based study provided a first indication that the antiasthma drug montelukast use might be associated with a lower risk of recurrent cardiovascular events[111].

### *1.2.8 The role of epigenetics in aortic valve stenosis*

Age related epigenetic modifications leading to aberrant global hypomethylation pattern in atherosclerosis has been proposed[112]. These functional changes representing overall genomic instability without structural mutations may result in alterations in certain gene transcriptional activity, contributing to increased atherosclerotic burden with its functional consequences. Epigenetic modifications including histone modifications, DNA methylation, small and non coding RNAs, chromatin architecture in addition to other transcriptional regulations ultimately regulate gene activity and expression. DNA methylation is linked to the transcriptional silencing or activation of certain genes. DNA methylation is predominantly found in CpG sites, which tend to cluster in regions of large repetitive sequences. The DNA methylation status is critical for the control of the transcriptional silencing/activity and the chromatin stability. For exemple, hypermethylation of the repetitive sequences in combination with histone modifications can result in condensation of the chromatin structure leading to suppression of certain gene activity. DNA methylation has been linked to several tumorgenesis in human through hypomethylation leading to activation (reactivation/restoration) of certain oncogenes[113, 114]. VICs, the dominating cell type of aortic valves, are responsible for the maintenance of valve integrity. However, this cell population is highly plastic demonstrating different phenotypic features both under physiological[6] and pathophysiological conditions toward bone-producing osteoblast-like cells[115]. The mechanism behind this phenotypic transdifferentiation process of VICs has not been fully explored yet. However, Miller et al.[116] proposed an assessment of epigenetic modifications in aVICs, which could be one possible mechanism beyond a sustained myofibroblast activation state.

### *1.2.9 The role of oxidative/nitrosative stress in aortic stenosis*

In the presence of established cardiovascular risk factors the initiating cellular process in aortic valve similar to that existing in atherosclerosis is the endothelial dysfunction[13]. Increased oxidative stress burden might play a critical role and contribute to stenosis progression. Previous findings provided evidence for increased levels of superoxide and $H_2O_2$ in the calcified regions of stenotic aortic valves, which was not counterbalanced by antioxidant enzymes, indicating dysfunctional protection[117]. Nitric oxide acts under physiological conditions as a potent anti-inflammatory[118], anti-apoptotic[119], and vasodilatator agent[120] and may protect against valvular calcification. However, a disturbed vascular redox balance results in eNOS (endothelial NO synthase) damage and uncoupling of oxygen activation from l-arginine conversion[121]. Uncoupled eNOS generates ROS rather than NO.

In addition, oxidative stress causing altered intracellular signaling, gene expression and rise in intracellular [$Ca^{2+}$], may lead to DNA-injury with subsequent activation of the nuclear enzyme poly(ADP-ribose) polymeras (PARP)[122]. DNA strand breakage through hydroxyl radical, nitroxyl anion and peroxy-nitrite (reactive oxidant formed from the reaction of nitric oxide and superoxide) is the obligatory trigger of PARP activation. PARP consists of a family of enzymes which catalyses poly(ADP-ribosyl)ation of DNA-binding proteins. Seven isoforms have been identified. PARP-1 is the best characterised member, and works as a DNA-damage nick sensor protein using beta-NAD(+) to form polymers of ADP-ribose[123]. Previous studies showed increased oxidative and nitrosative stress in response to myocardial infarction i.e. increased nitrotyrosine staining, oxidative stress, antioxidant depletion, increase in oxidative markers, increase in circulating nitrite/nitrate formation (breakdown products of nitric oxide), and has been demonstrated in the late stage of reperfusion[124]. Overactivation of PARP in oxidatively stressed cells can lead to suppression of cellular metabolic function with subsequent depletion of cellular NAD+ and ATP content, mitochondrial dysfunction and ultimately necrotic cell death and organ dysfunction[125], representing a final common pathway involved in the pathophysiology of many cardiovascular diseases. However, the pathogenetic role of PARP-1 activation was not investigated in aortic valve stenosis.

### *1.2.10 Extracellular matrix remodeling*

The quantity and quality of ECM together with the VICs are responsible for the maintenance of competency of the AV throughout a lifetime. However, under pathological remodeling process a number of ECM proteins normally occurring in skeletal bone are present following the pathological matrix degradation due to increased tissue levels of cysteine proteases (cathepsins), MMPs secreted by aVICs and infiltrating immune cells. These processes are collectively associated with loss of the valve integrity and function. The increased tissue levels of elastolytic enzymes, such as cathepsin S, K, and V in calcified aortic valves lead to elastin fragmentation which reflects a disturbed balance between ECM synthesis and degradation[126]. In parallel, macrophages in human atherosclerotic lesions overexpress cathepsin L, leading to necrotic core formation with subsequent plaque instability[127]. In the context of AS, this process contributes to the stiffening of valvular cusps and represents a *nidus* together with apoptotic cells and matrix vesicles for subsequent calcification[80]. To further confirm the active influence of cathepsins in calcification process, a previous observation demonstrated abolished arterial and aortic valve calcification in cathepsinS knock-out mice with chronic renal disease[34]. Simultaneously with extensive ECM remodeling non-collagenous bone associated proteins are assumed to have a critical role in further compositional changes of ECM. Related to that, several osteoblast markers such as osteonectin, bone sialoprotein, OPN: a multifunctional glycophosphoprotein are involved in both the inflammation and biomineralization and accumulate at the sites of calcifications. Earlier in vivo investigations showed that OPN had an active inhibitory effect on mineral deposition and promotes regression of ectopic calcification[128]. However, later observations including studies assessing circulating plasma levels of OPN indicated that the presence of OPN rather is associated with aortic valve calcification and the degree of stenosis[129]. This functional implication of OPN is probably coupled to its role as an indicator of active inflammation, which is observed in patients with acute coronary syndrome , unstable coronary plaque, and after PCI (percutaneous coronary intervention) representing a possible predictor of accelerated atherosclerosis[130]. In addition, the known posttranslational modifications of OPN (phosphorylation) can modulate its biological function. OPN is a substrate for TRAP, highly expressed by osteoclasts. Partial dephosphorylation of OPN by the action of TRAP regulates the osteoclast's attachment ability to the bone surfaces and their migration to new resorptive sites[131]. Several studies performed in patients with AS revealed high circulating plasma levels of OPN compared with controls[129, 132-135].

### *1.2.11 In situ calcification*

Descriptive studies demonstrated upregulation of molecular calcification markers in calcified aortic valve tissue including Runx2/Cbfa1, a central transcriptional regulator of osteoblastogenesis[70], osteocalcin[115], bone-morphogenetic protein 2 (BMP-2)[11], and the active

influence of RANKL/RANK/OPG-axis regulating osteoclast differentiation[136], activation and survival[137]. Moreover, bone morphogenetic protein related and Wnt/β-catenin signaling pathway have been observed in calcified regions of excised human aortic valves[13, 138] and in experimental animal models[66, 116, 139].

Accumulation of calcium into the valvular tissue occurs via several different processes: 1) caspase-dependent apoptosis, 2) osteoblastic differentiation of VICs [116], 3) due to enhanced proteolytic activity leading to production of elastokines favoring calcification, 4) through homing of circulating cells with hematopoietic origin possessing osteogenic properties[140] and 5) passive accumulation of calcium.

The intact well-functioning aortic valve is gradually replaced by pronounced calcification containing elements of lamellar bone with microfactures[70], apoptotic cores[86-88] with preceding endochondrial bone tissue[70, 89] resembling to that existing in skeletal bone which is the key feature in advanced stages of the stenotic lesions with subsequent hemodynamic consequences. Histopathologically the early phase demonstrating valvular thickening is characterized by inflammation[73, 141, 142], disruption of the basement membrane, presence of extra-and intracellular lipids[143] and infiltrating immune cells and fine stippled mineralization[68] whereas in the end-stage disease heterotopic bone formation takes place, which is an actively regulated form of bone formation[70] containing large amount of calcium. In addition, as earlier observations demonstrated, the extent of valvular calcification is a strong independent predictor of outcome[47] and correlates with the stenosis severity[144]. Molecular imaging technique provided evidence for inflammation-dependent calcification paradigm already in the early stage of the disease[80, 145].

Heterotopic bone formation at ectopic sites occurs, when 1) specific group of cytokines with pro-osteogenic potential mediate osteoinduction, 2) the surrounding milieu favors osteogenesis[146] and 3) the aortic valves own structural cells serve as a prerequisite with a propensity to transform into bone producing osteoblast cells due to their phenotypic plasticity, besides the presence of circulating osteogenic precursors, CD45(+)[140]. The latter study provided evidence that osteogenic cells in the blood home to sites of vascular injury and are associated with heterotopic ossification in heart valves[140]. This cell population identified as a bone marrow–derived type 1 collagen$^{+}$/CD45$^{+}$ subpopulation of mononuclear adherent cells that are present in early preosseous (fibroproliferative) lesions of heterotopic ossification[147]. Additionally, valvular endothelial cells also contribute to the osteoprogenitors through endothelial-mesenchymal transition[148]. The heterotopic bone has all the morphologic and biochemical characteristics of orthotopic bone, is subjected to the intriguing ability to generate the formation of bone marrow[146, 149].

Collectively, heterotopic ossification characterized as a reactive proliferative tissue with features resembling to that existing in skeletal bones, however in chronically damaged soft tissue. Furthermore, this ectopic bone exhibits higher remodeling rate compared to that existing in the normal bone tissue and demonstrates more than twice the number of osteoclasts compared to normal age-matched control[149] representing uncoupling of bone formation from resorption with decreased osteoblast and increased osteoclast number and activity. The serum alkaline phosphatase level, a marker of osteoblast activity, is elevated early in the course of heterotopic ossification but returns to normal levels as maturation proceeds[150].

However, according to a detailed histological observation by Mohler et al. including 347 surgically excised human valve specimens (256 aortic, 91 mitral valves) the prevalence of dystrophic calcification was 83%, mature lamellar bone with hematopoietic elements and active bone remodeling was found only in 13% along with dystrophic calcification. Interestingly, high prevalence of microfractures was present, 92% of all the examined valves with ossification[70].

Taken together, almost all previous observations conducted in stenotic AV tissue focused on the molecular mechanism of active bone formation (heterotopic bone formation), although this part contributes only to a small amount of local calcification. In addition, local accumulation of elastin degradation products, as a result of enhanced proteolytic activity triggers the calcification partly through stimulation of phenotypic transdifferentiation of VICs and on the other hand through the elastins increased polarity to calcium together with apoptotic bodies and matrix vesicles promotes foci for hydroxyapatite nucleation[145].

Clinical studies and observations from experimental models suggested inflammation, as a common cause with reciprocal regulation on vascular and valvular calcifications and on bone osteogenesis[151].

The bone remodeling in calcified aortic valves has its origin in microfractures at multiple sites of the valve, at which osteoclasts are the first cells recruited, followed by osteoblasts, together building up a complex system: a BMU. This unit works as a spatially and temporally controlled team of bone-resorbing osteoclasts (10-20 cells) and bone-forming osteoblasts, as team of 1000-2000 cells[152].

An interesting advanced mathematical model described the evolution, the spatio-temporal dynamics of BMU, and the interplay between the BMU geometry and its regulatory cytokine system containing RANKL, RANK and OPG.

Bone remodeling in aortic valve tissue proceeds asynchronously at multiple sites in response to multiple microfractures due to cyclic hemodynamic forces. According to this elegant applied mathematical model[152], as the time progress, osteoclasts continue to move in form of a cutting cone along the damage lines, followed by osteoblasts replacing the resorbed bone. In response to RANKL field which exhibited the highest concentration at the site of microfracture in front of the BMU, osteoclasts were able to move as a well-confine cutting cone, whereas OPG reached its highest concentration back of the BMU. These concentration differences lead to development of RANKL/OPG gradient which finally determinates the rate and direction of BMU progression[152]. In contrast, there is a little conclusive information on the osteoclasts activity, their relation to stenosis severity and the interplay between osteoblasts and osteoclasts. Mohler et al. described first multinucleated cells within the resorption spaces (Howship`s lacunae) in the calcified part of the valvular tissue.

However, their role is not well investigated. Other studies confirmed the presence of osteoclasts[4, 153] through γ-GT positivity and co-expression with CD 68[154], confirming their hematopoietic origin. The latter study demonstrating through negative correlation to the valve calcium content and the stenosis severity suggested that osteoclasts could decrease the mineral content of the valve and thus limit the progression of valvular stenosis.

To date, no previous study assessed the spatial distribution of bone turnover containing genes with osteoinductive and osteoresorptive potentials obtained from different parts of calcified aortic valve tissue and their relation to the clinical stenosis severity. In addition no previous observation on the posttranslational phosphorylation level of OPN on osteoclast function was performed in aortic valve specimens, which regulates the extent of degradation. Furthermore, according to the previous description on the extent and relationship between dystrophic calcification and active lamellar bone formation in excised AV tissue[70], one can speculate that uncoupling due to decreased osteoblast population and enhanced osteoclast burden in calcified aortic valve lesions exists.

## 1.3 EXPERIMENTAL ANIMAL MODELS

To date, only aging swine develop valvular sclerosis without initiating factor[155], while other species require special diets or genetic mutations[156]. However, even the animal models do not recapitulate fully the natural course of AS occurring in human, but contribute to better understanding of the underlying mechanisms at tissue and cellular level. The major limitation using experimental models of AS that few of them develop hemodynamically significant valvular lesion. To date, two experimental animal models have been identified in mice, which consistently develop hemodynamically significant AS[116]: 1) the $LDL^{-/-}$ /$apoB^{100/100}$/$Mttp^{fl/fl}$/$Mx1\text{-}Cre^{+/+}$=Reversa mice, in which reducing plasma lipid levels by genetic inactivation of the mttp gene with early aortic valve disease normalizes the oxidative stress, reduces pro-osteogenic signaling, and halts the progression of AS[66], 2) $Egfr^{wa2/wa2}$ (hypomorphic Epidermal growth factor receptor allele), demonstrating a phenotype with significantly thicker aortic valve cusps with subsequent elevated transvalvular pressure gradients [157].

## 1.4 TREATMENT OPTIONS

As to date no pharmacological treatment option has been shown to halt the hemodynamic progression of AS, surgical valve replacement is still the standard therapy for this disease.

However, many patients not suitable candidate for surgery because of multiple co-morbid conditions with high anticipated perioperative risk and therefore they are subjected to transcatheter valve replacement[158]. In this part of the introduction section an overview will be given covering previously proven pharmacological interventions in the context of AS.

### *1.4.1 Statins*

Based on promising results of lipid lowering treatment in experimental animal models of AS[65, 66, 159] and on those obtained in numerous retrospective observational studies[38, 156, 160-162] where statin treatment was used in primary or secondary prevention, to date four large randomized trials[163-165] are published, however with an overall neutral results on the hemodynamic progression of AS, except for one[166]. The latter study (RAAVE)[166] was en prospective study with open label design, which provided the first clinical evidence for beneficial effect of targeted HMG-coenzyme A reductase inhibitor treatment in terms of hemodynamic retardation in patients with asymptomatic moderate AS and known elevated plasma LDL-levels. A recently published meta-analysis on randomized lipid trials on patients with AS examined the effects of treatment on AS progression and clinical outcomes[167].The authors concluded, that the current data do not support the hypothesis that statin therapy reduces AS progression.

The major criticisms against the published interventional trials using statin treatment are:

1) The trials were designed according to the traditional vascular trials using vascular and valvular end points[163, 164, 168].

2) The late initiation of statin treatment in the disease course[163].

3) The short duration of follow-up used in the studies above, given that aortic stenosis develops over long period of time.

4) There are differences between the trials regarding the enrollment criteria, statin medication and timing of therapy, which all do not offer direct comparisons between the trials.

Although, the published interventional statin-studies did not demonstrate hemodynamic retardation of AS, in contrast statistically significant improvement in vascular end points were observed, suggesting that atherosclerosis and AS are two different disease entities.

As a further explanation of the observed neutral results, two fundamental differences in valvular versus vascular biology has to be mentioned: the different biological direction of the valvular and vascular disease development with a dominating inward direction causing occlusion of the vascular lumen over time, whereas the direction in which LDL affects the valve is upward direction along the y-axis corresponding of the aortic valve area[14].

There are more additional facts questioning the effectiveness of statin treatment in AS. First, a report described the paradoxical effects of statin treatment in vitro cell culture model of primary isolated porcine VICs reporting limited dystrophic calcification but paradoxically increased bone cell calcification[169], analogous to “statin paradox”[156] reported in the field of osteoporosis research. Second, in line with this observation, the partial therapeutical failure of statins may be due to the induction of the phosphorilated ERK (Extracellular-regulated kinases), which in turn triggers the proliferative degeneration and calcification process[170], whereas the inflammation associated genes exhibited downregulated expression levels in statin-pretreated human aortic valve specimens compared to untreated ones[171]. Third, statins are known as DNA methyltransferase inhibitors, and thus leading to demethylation of the BMP-2 promoter with subsequent enhanced BMP-2 expression in colon cancer cells, which is beneficial in those cells because of the BMP-2’s tumor suppressor properties[172]. Collectively, this statin induced shift of BMP-2 transcriptional profile through epigenetic modifications however has no beneficial effect in calcific AS, rather leading to enhanced local calcification. And finally, statins have anabolic action on bone by increasing bone formation and improving fracture repair[173]. They suppress cell apoptosis partly through TGF-β -Smad3 pathway in osteoblastic cells[174].

### *1.4.2 The Renin-Angiotensin System blockade therapy*

In human AV specimens pressure overload increases the renin-angiotensin system at early stages[175], promoting development of left ventricular hypertrophy and fibrosis leading to myocardial contractile dysfunction. This notion was further supported by histopathologically demonstrated presence of angiotensin-converting enzyme (ACE), angiotensin II and angiotensin II type 1 receptor in which suggested that ACE may participate in the valvular lesion development[67]. The latter observation suggested that ACE inhibitor use may be associated with reduced calcium accumulation into the valvular tissue, but no effects to retard the hemodynamic progression rate of AS[67]. In addition, a recently published retrospective analysis in a japanese cohort (Japanese Aortic Stenosis Study) identified 2 risk factors coupled to stenosis progression in early stage of the disease: 1) no use of ARB and 2) use of warfarin which increases the calcium content of the valves[176, 177]. Furthermore, a recently published large retrospective observational trial including a total of 2117 patients with AS suggested that ACE-inhibitor/Angiotensin receptor blockade (ARB) is associated with improved survival, lower all-cause mortality and cardiovascular event rate[178]. The authors concluded, that a part of the observed beneficial effects might be due to the effects of ACE-inhibitor/ARB on the valvular, ventricular or on systemic level.

### *1.4.3 Bisphosphonates*

Recently published restrospective analysis including patients with aortic valve stenosis and co-existing osteoporosis, where nitrogen-containing bisphophonate treatment for osteoporosis was independently associated with slower progression rate of stenosis severity[179-181] suggested the active influence of osteoclasts in disease progression. These results hence indicate that local induction of osteoclast apoptosis in the calcified aortic valve might have a beneficial hemodynamic effect. In addition to prevent osteoclast-mediated bone resorption, bisphosphonates are potent inhibitors of farnesyl-pyrophosphate synthase, an enzyme located distal of HMG-coa in the mevalonate pathway. By the action of nitrogen-containing bisphosphonates the amount of isoprenoids, such as fanesyl-pyrophosphate and geranylgeranylpyrophosphate decreases, explaining their “statin like pleiotropic effects” that decreases inflammation, matrix degradation, and proliferation[182]. However, the beneficial effects of the bisphosphonate treatment with decreased cardiovascular calcification were observed in elderly, in contrast the prevalence of valvular, aortic and coronary artery calcification increased in younger women with an age of <65 years[183]. In addition, a most recently published retrospective analysis with a hitherto largest patient population of 800 female patients with an age of >60 years failed to confirm the previously reported promising results[184].

# 2 AIMS

1. To explore the pathogenetic role of the leukotriene-pathway in aortic valve stenosis, and its relation/contribution to the stenosis severity (Study I)

2. To characterize the transcriptional profile of Poly-ADP-ribose polymerase 1 in bicuspid and in tricuspid aortic valves, respectively and its relation to the clinical stenosis severity (Study II)

3. To further extend the $LTC_4$ intracellular effects in vascular smooth muscle cell, a mesenchymal cell type similar to valvular interstitial cells (Study III)

4. To investigate the role of epigenetics in phenotypic transdifferentiation of the valvular interstitial cells (Study IV)

5. To study the role of genes with osteoinductive and osteoresorptive functions focusing on the roles of osteoclasts in aortic valve tissue and their relation to clinical stenosis severity (Study V)

# 3 MATERIALS AND METHODS

All experiments included in the present thesis were performed on human materials or on cells of human origin. The corresponding papers are referred to with Roman numerals (I-V).

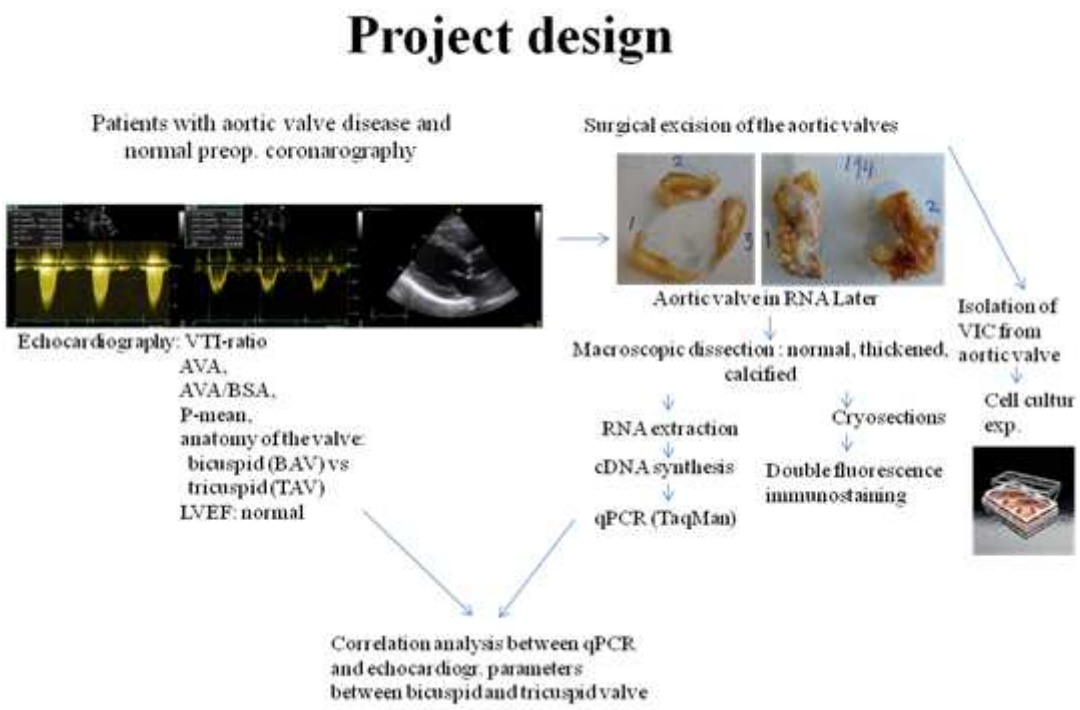



*Figure 2. Overview of the study design.*

*Patient cohort, clinical data*
*Patients*
Human aortic valves for RNA extraction were obtained from 79 patients undergoing aortic valve replacement surgery. After the exclusion of 11 patients due to insufficient RNA quality, 68 patients were included in the subsequent analysis (48 male and 20 women, mean age 65.9± 13.4 years) (Table 1). For cell- and organ culture experiments, aortic valves from an additional 12 patients were used (**paper I**). Only patients with a normal preoperative coronary angiogram and no history of rheumatic heart disease were included in the study (Figure 2).

*Echocardiography*
All patients underwent two-dimensional transthoracic Doppler echocardiography using a Philips IE33 system (Philips Medical Systems, Andover, MA).The severity of aortic valve stenosis was based on, 1) the aortic valve area (AVA, $cm^2$) calculated using the continuity equation, 2) AVA indexed for body surface area (AVA/BSA, $cm^2/m^2$), 3) the ratio of the VTI in the left ventricle outflow tract to the VTI in the aortic valve and 4) the mean transvalvular pressure gradient according to the current guidelines[42, 43, 185]. The echocardiographic assessment of the aortic valve as bicuspid or tricuspid was confirmed intraoperatively.
Based on the echocardiographic assessment, the study population was categorized into 5 diagnosis groups: 1) normal aortic valve (pathology of the aortic root and/or the ascending aorta without valve dysfunction in which valve-sparing surgery could not be performed according to perioperative surgical decision), n=4; 2) combined aortic vitium, i.e., increased transvalvular pressure gradient without fulfilling the criteria for severe aortic stenosis and the presence of aortic valve regurgitation, n=7; 3) isolated aortic regurgitation, n=11; and finally, isolated, severe aortic stenosis with either a 4) bicuspid, n=23 or 5) tricuspid, n=23 valve (Table 1).

*Quantification of the extent of valvular calcification: Aortic valve calcification (AVC) scoring (paper V)*

In real time short-axis (SAX) view a single score (from 1-5) was assigned to each aortic leaflets 1: normal leaflet, 2: thickened with no evidence of calcification, 3: mild calcification (<1/3 of leaflet area), 4: moderate calcification (1/3-2/3 of leaflet area) and 5: severe calcification (>2/3 leaflet area).

Table 1. Patient characteristics (**paper I**)

| | **Aortic root pathology without aortic valve dysfunction** | **Combined aortic vitium** | **Aortic regurgitation** | **Aortic valve stenosis** | | **Overall *P*-value** |
|---|---|---|---|---|---|---|
| | | | | Bicuspid aortic valve | Tricuspid aortic valve | |
| **No. of patients** | 4 | 7 | 11 | 23 | 23 | |
| **Male, n (%)** | 2 (50%) | 6 (86%) | 11 (100%) | 16 (69%) | 13 (56%) | |
| **Age, y (range)** | 66.6 (55.4 - 76.5) | 59 (37.7-82.5) | 61 (31.5-74.6)* | 64.4 (28.5-82.4)* | 76.2 (57.9-87.1) | 0.002 |
| **BMI, kg/m²** | 25.3 (21.5-35.9) | 24.7 (24.2-31) | 25.9 (18.1-28.4) | 26.9 (19.2-34.7) | 26.3 (21.6-37.4) | 0.228 |
| **AVA, cm²** | 2.1 (1.2-3.2)† | 1.3 (1.2-2.5)† | 2.6 (1.7-6.8) | 0.76 (0.47-1.2) | 0.72 (0.45-1) | <0.001 |
| **AVA/BSA, cm²/ m²** | | 0.83 (0.67-1.2) † | | 0.4 (0.3-0.6) | 0.38 (0.25-0.58) | 0.003 |
| **VTI-ratio** | 0.4 (0.27-0.56) | 0.26 (0.21-0.48) | 0.49 (0.38-0.91) † | 0.2 (0.16-0.28) | 0.2 (0.14-0.37) | <0.001 |
| **Aorta Vmax, m/s** | 2.2 (1.8-2.9)‖ | 4.1 (1.8-4.6) † | 2 (1.6-3.6) † | 4.5 (3.4-5.7) | 4.5 (4-5.8) | <0.001 |
| **P-mean, mm Hg** | 12 (7-20) † | 40 (9-48) † | 9 (5-30) † | 51 (27-85) | 49 (37-93) | <0.001 |
| **Left ventricular EF, %** | 52% (37-64) | 59% (47-68) | 59% (38-75) | 61% (44-78) | 64% (42-75) | 0.185 |
| **LVEDD, mm** | 50 (46-55) | 51 (44-63) | 57 (63-92) † | 49 (36-63) | 47 (37-57) | <0.001 |

*Left ventricular EF: left ventricular ejection fraction; LVEDD: left ventricular end diastolic diameter. Values are medians and ranges unless otherwise stated. *P<0.05 compared with tricuspid; †P<0.05 compared with tricuspid and bicuspid; Kruskal-Wallis ANOVA on ranks followed by Dunn's all pairwise post hoc testing.*

*Blood sample collection*

Blood was drawn from an antecubital vein into EDTA tubes from all the patients with AS (**paper V**), put on ice and centrifuged within 30 min. Plasma was stored at -80C until analysis of bone-related circulating biomarkers. Serum creatinine, lipid profile, HbA1c, and high sensitive CRP were measured using well-established methods routinely used in clinical practice.

*Macroscopic dissection*

*Preparation and macroscopic dissection of aortic valve specimens*

Immediately after surgical removal, valves were immersed in RNA Later (Qiagen) and stored at 4°C until transport to the laboratory. Macroscopic dissection was performed in the RNA Later solution, dividing each valvular cusp into: 1) normal areas defined as non-calcified, smooth, pliable, and opalescent; 2) thickened areas; and 3) calcified areas, as demonstrated on Figure 3. From each class, 1 sample from each cusp was frozen at -80°C until RNA extraction and another was used for histological analysis. The macroscopic definitions were verified by histology in a subset of preparations (n=89) using eosin and hematoxylin, Masson trichrome and Alizarin red as indicated in Supplemental Fig 1 in **paper I**. Furthermore, the

degree of calcification detected macroscopically in each cusp using morphometric analysis was in agreement with preoperative echocardiographic scoring of calcification (Figure 3).

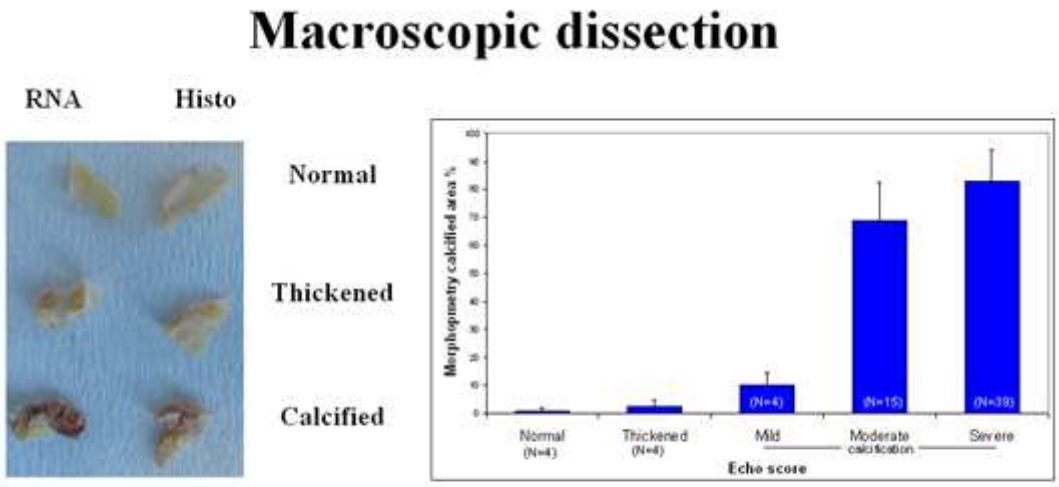



*Figure 3. The left panel shows examples of adjacent tissues from stenotic aortic valves used for RNA extraction and histology, respectively. The right panel shows the correlation between gross anatomy (macroscopic dissection) and the aortic valve echocardiographic calcification scoring.*

*RNA isolation, cDNA synthesis, Taqman real-time qPCR, microarray analysis*
*RNA extraction and quality assessment*
In all papers included in this thesis total RNA was isolated from either whole tissue, representing the various parts (normal, thickened, and calcified) from each aortic valve cusp or from cell culture experiment using the RNeasy Lipid Tissue Mini kit (Qiagen). RNA concentrations were measured spectrophotometrically at 260 nm (A260/280 nm, Agilent Technologies, Palo Alto, CA).
The quality of the RNA was analyzed on a 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) using RNA 6000 NANO chips to assess the RNA Integrity Number (RIN), as described[186]. Consequently, of the 79 valves that were collected for RNA analysis, 11 patients were excluded due to insufficient RNA quality, and the gene expression data for 311 preparations from 68 patients were used for further analysis (**paper I**). On average, 4-5 preparations were examined for each aortic valve. In subsequent analyses, a mean value of each tissue category (i.e. normal, thickened, and calcified, respectively) was calculated for each individual.

*mRNA and DNA extraction in paper IV*
VICs were harvested for RNA and DNA isolation using the AllPrep RNA&DNA, Qiagen according to the manufacturer's instructions. DNA and mRNA extraction from non-calcified (thickened) and calcified parts of valvular tissue were performed using DNeasy and RNeasy kits (Qiagen), respectively.

*TaqMan real-time PCR*
In all papers included in this thesis, gene expression analysis was performed by using TaqMan real-time qPCR. First-strand cDNA was synthesized from 0.5 µg RNA (Superscript II, Invitrogen, Carlsbad, CA) with random hexamers according to the manufacturer`s instructions. Quantitative TaqMan PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Bisosystems) with primer/probe pairs that were obtained using Assay-on-demand™ from Applied Biosystems (Supplemental Table 1 in **paper I**); the reactions

contained 5 µl cDNA which was diluted to 1.5 ng/µl and 5 µl TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems, Foster City, USA).
The following primer/probe pairs, Assay-on-demand™ were used from Applied Biosystems in **paper II**: Cyclophilin A=PPIA: Hs99999904_m1; poly (ADP-ribose) polymerase 1=PARP-1: Hs00911369_g1, and in **paper V**: BGLAP: Hs01587813_g1; BMP-2: Hs01055564_m1; BMP-6: Hs01099594_m1; Runx2/Cbfa1: Hs00231692_m1; TNFSF11=OPG: Hs00243522_m1; TRAP: Hs00356261_m1; SPP1: Hs00959019_m1; RANK: Hs00187192_m1; RANKL: Hs002433522_m1. Results were normalized to the expression levels of PPIA.

*Transcriptional changes in VICs (paper I, II, IV) and in SMCs (paper III)*
To evaluate transcriptional changes induced by either $LTC_4$ or by different cytokines, such as TGF-β1, IL-1β, VICs (passages 2-3) were seeded in 6-well plates ($10^5$ cells per well). After 24 hours serum-starvation, VICs were incubated in the absence or presence of $LTC_4$ at either 1 or 10 nM, and collected for RNA extraction and determination of expression levels of BMP-2, BMP-6, and the Runx2/Cbfa1 using TaqMan real time PCR (see Suppl Table 1 for primer/probe references in paper I). The same set-up of cell culture experiment was used in **paper II** to evaluate the transcriptional changes of PARP-1 induced by various cytokines, such as TGF-β1 (10 ng/mL), IL-1β (10 ng/mL) and $LTC_4$ (1nM).
In **paper III** the same procedure was used for total RNA extraction, RNA quantity and quality assessment from SMCs as previously described. After cDNA synthesis qTaqMan PCR was performed with primer/probe pairs that were obtained using Assay-on-demand™ from Applied Biosystems for human $CysLT_1$ (Hs00272624_s1) and PAI-2/SERPIN B2 (Hs01010736_m1). Levels of mRNA were normalized to expression levels of PPIA, which previously has been determined as an appropriate housekeeping gene in these cells.

*Microarray Analysis in paper III*
Microarray analysis experiments were performed on RNA derived from three separate SMC culture experiments, using either Agilent one-color whole human genome (44K) kit (Agilent Technologies, Redwood city, USA; n=2) or the Affymetrix Human Genome U133 Plus 2.0 array (n=1). Microarrays were analyzed with the Agilent high resolution microarray scanner. Data was subsequently uploaded to GeneSpring GX10 (Agilent technologies), and analyzed using advanced analysis workflow for the Agilent one-color arrays. The set of data was normalized according to recommendations by GeneSpring for one-color arrays. (http://www.chem.agilent.com/cag/bsp/products/gsgx/manuals/GeneSpring-manual.pdf).
Variability between chips was accounted for by applying a shift to the 75th percentile (dividing all measured signals by a 75th percentile value). Per-gene normalization was performed by bringing the baseline to the median of all samples. Probe sets were firstly filtered by confidence of detection, where genes that were not confidently detected in any sample were excluded from further analysis.
Further filtering based on expression discarded any genes where less than 100 percent of samples in either the relapse or diagnosis condition had expression values below the $20^{th}$ percentile. The most differentially expressed genes, defined as those with an uncorrected p-value of <0.05 and demonstrating a fold-change in expression of 2.0 or greater, were selected for analysis. The list of 90 genes generated was subsequently compared to data from the Affymetrix arrays and the genes of interest were verified in terms of direction of regulation. Genes meeting all these criteria contained 45 genes, which were submitted to Ingenuity® Pathway Analysis for prediction of canonical pathways and functional gene networks affected by the significant differential expression of these genes.

*Analysis of the methylation status of the 5-LO promoter region*
*DNA hypomethylation by 5-Aza-2'-deoxycytidine (AdC) on VICs in paper IV*
Cells were seeded into 6-well plates and incubated for 96 hours in the absence or presence of the hypomethylating agent, 5-Aza-2'-deoxycytidine (Sigma) at different concentrations (100 nM-10 µM). The cell culture medium and AdC were replaced every 24 hours.

*Analysis of the DNA methylation status of the 5-LO promoter region in paper IV*
Genomic DNA extracted from VICs and valve tissue were further analyzed using the DNA Methylation Enzyme Kit (SA Biosciences) containing methylation sensitive -and methylation dependent restriction enzymes followed by SYBR green-based Methyl-Profiler™ qPCR Primer Assay for Human ALOX5 (CpG Island: 13671; Related Refseq: NM_000698 (ALOX5); TSS Position: 45189635; TSS Orientation: Forward; SA Biosciences) to analyze the methylation status of CpG islands in the promoter region of 5-LO.
Using methylation sensitive- and methylation-dependent restriction enzymes, which selectively digest the unmethylated and the methylated DNA, respectively, allowed obtaining the methylation density present in the region studied. The change in Ct between the treatments using the double digest and the mock digest served as the average analytical window. The resistant fraction after each treatment was assessed by SYBR green-based Methyl-Profiler™ qPCR, using primers that specifically flank the region of interest of the human ALOX5 gene. The assay covers one CpG island in the promoter region and the PCR product size is 157 bp. The number of CpG sites in that island is 117 (http://genome.ucsc.edu/cgi-bin/hgc?hgsid=244484841&o=45188724&t=45190162&g=cpgIslandExt&i=CpG%3A+117).

*Immunohistochemistry*
In all paper double fluorescent immunostaining was used on either frozen sections of valvular tissue, human carotid endarterectomy samples or on primary isolated VICs and SMCs for simultaneous detection of two proteins of interest.
Transversal cryosections (10 µm thick) were cut, oriented from the cusp base to the free edge, and fixed in acetone. Double immunofluorescent stainings were performed using polyclonal rabbit anti-human $CysLT_1$ receptor (Cayman Chemical Company) or 5-LO (Life Science Bioscience) and monoclonal mouse anti-human vimentin or CD68 (Dako) (**paper I, IV**), polyclonal rabbit anti-human PARP-1 (Abcam) (**paper II**), α-smooth muscle actin (from DAKO) (**paper III**), and monoclonal mouse antihuman antibody for TRAP (**paper V**) as primary antibodies. Isotype-specific either Texas Red or Alexa Fluor 488-conjugated secondary antibodies (Abcam) were used and the nuclei were counterstained with DAPI (Vector). Images were captured with confocal microscope Leica DMI.

*Cell biological experiments*
*VIC isolation (paper I, II, IV)*
Aortic valve samples in cell culture medium were cut into small cubes (1-2 mm in each dimension) using a scalpel and placed onto culture dishes to dry for approximately 15 min under sterile conditions. Then, the valvular pieces were covered with DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37ºC and 5% $CO_2$.
The culture medium was changed 3 times per week, and the outgrowth of valvular myofibroblasts was evaluated during 2-3 weeks. At this point, outgrown myofibroblasts were detached and reseeded for measurement of intracellular calcium concentrations, mitochondrial membrane potential and ROS, calcified nodule formation and transcriptional changes.

*Calcium measurements (paper I, III)*
Free cytoplasmic [$Ca^{2+}$] was measured using the fluorescent calcium indicator Fluo-3 (Molecular Probes/Invitrogen) and confocal microscopy (Bio-Rad MRC 1024, attached to a Nikon Diaphot inverted microscope with a Nikon Plan Apo 40× oil immersion objective (NA 1.3)). The myofibroblasts were loaded with the cell-permeant acetoxymethyl ester (AM) form of Fluo-3 (Fluo-3 AM; 5 µm) for 30 minutes at $37^0$C, followed by washout with $CO_2/O_2$ (5/95%)-bubbled Tyrode solution with the following (in mmol/l): 121 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $NaH_2PO_4$, 24 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose. In some experiments, $Ca^{2+}$-free Tyrode solution was used, wherein $CaCl_2$ was replaced with 5 mM EGTA. Fluo-3 fluorescence was stimulated by excitation with 488 nm laser light while measuring the emitted light through a filter that allowed wavelengths >515 nm to pass. A

stack (z-dimension) of 15-20 confocal images, each approximately 0.5 μm thick, was acquired at regular intervals during the experiment. Fluorescence intensity was measured in the same cell regions at each time point. All experiments were performed at room temperature (~22$^0$C). In **paper III** similar calcium signaling experiments were performed on SMCs, as in **paper I**. In brief, SMCs were incubated for 48 hours in the absence or presence of LPS (10 μg/mL) followed by washout and then loaded with the fluorescent calcium indicator Fluo-3. The cells were subsequently stimulated with $LTC_4$ (1 μM, 30 min at room temperature) in either Tyrode's solution (the composition was the same as it was used in paper I) or Tyrode's solution containing the $CysLT_1$ receptor antagonist MK571 (1 μM). Changes in [$Ca^{2+}$] were recorded using a BioRad MRC 1024 confocal microscopy as previously described.

*Measurement of mitochondrial membrane potential and reactive oxygen species (paper I)*
To measure mitochondrial membrane potential (ΔΨm), the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE; Invitrogen/Molecular Probes) was used. Because TMRE accumulates in the negatively charged mitochondrial matrix in proportion to the electrical potential over the inner mitochondrial membrane; the change in fluorescence reflects the changes in ΔΨm. The myofibroblasts were loaded with TMRE (~1 μM) for 20 min at 37$^0$C, followed by washout. Confocal images of TMRE fluorescence were obtained at 568 nm excitation/585 nm emission. At the end of the experiment, the myofibroblasts were exposed to the mitochondrial uncoupling agent carbonyl cyanide-p trifluoromethoxyphenylhydrazone (FCCP; 1 μM), which depolarizes the mitochondria, allowing the dynamic range of the dye to be measured. Fluorescence was measured in the same cell regions at each time point. Changes in mitochondrial ROS (superoxide (O2*–)) production were monitored using the fluorescent indicator MitoSOX Red (Invitrogen/Molecular Probes), which accumulates preferentially in mitochondria and specifically detects the formation of O2 *–. Cells were incubated with MitoSOX Red (5 μM) for ~30 min at 37$^0$C, and experiments were started after the cells were washed with Tyrode solution. MitoSOX Red was measured at 488 nm excitation/>585 nm emission.

*Calcified nodule formation in primary culture of VICs*
For studies of calcification, primary cultures of human VICs were plated into 6-well cell culture plates with a cell density of $10^5$ cells/well in DMEM supplemented with 10% heat inactivated fetal bovine serum and antibiotics. In addition, β-glycerophosphat (10 mmol/L), $CaCl_2$ (1.5 mmol/L) and ascorbic acid (50μg/mL) were added to the cell culture medium to enhance calcification. The cell culture medium was changed every second day with $LTC_4$ added at different concentrations (1-100 nM). After two weeks, calcified nodule formation was evaluated by Alizarin red staining.

*Cell culture experiment in paper III*
Human coronary artery SMC purchased from Clonetics (Cambrex Bio Science, Walkersville, MD), were cultured in SmGM2 kit medium and harvested for experiments between passages 5 and 8. Cells were seeded in 6-well plates ($10^5$ cells/well) containing SmGM2 48 h before the respective treatment, which was replaced with Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% FCS (starvation medium) 24 hours before experiments. Subsequently, cells were incubated in the absence or presence of LPS (Sigma, 10 μg/mL), IL-6 (Peprotech, 20 ng/mL), IFN-γ (Peprotech, 20 ng/mL), or TNF-α (Peprotech, 10 ng/mL). Duration of LPS treatment was experiment dependent. Cells were treated with various substances across experiments, including $LTC_4$, $LTD_4$, and $LTE_4$ (Cayman Chem, Ann Arbor, USA; 1 μM), the CysLT1 receptor antagonist MK571 (Cayman; 1 μM), EGTA (Sigma, 5 mM), and BAPTA-AM (Invitrogen, 10 μM).

*ELISA (paper I, III, IV and V)*
Concentrations of cysteinyl-LTs were measured using an enzyme immunoassay (EIA kit from Cayman Chemicals) with a monoclonal antibody recognizing the total of all three cysteinyl-LTs ($LTC_4$, $LTD_4$, $LTE_4$) on conditioned media containing healthy, thickened and calcified parts of stenotic aortic valves (n=72, derived from 5 patients) after 24 hours

incubation at 37ºC in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (**paper I**). The quantitative measurements of concentrations of PAI-2 was carried out on cell lysates from untreated SMCs and SMCs treated with $LTC_4$ (1μM) for 24 hours using IMUBIND® PAI-2 ELISA kit (American Diagnostica GmBH) according to manufacturer's protocol (**paper III)**. In **paper IV**, to quantify the concentration of $LTB_4$ in supernatants from VICs, ELISA (Cayman Chemical) was used. In **paper V** plasma concentrations of human total OPG, MMP-9 (R&D Systems), BMP-2 (Boster Immunoleader), BMP-6, BGLAP, RANK, Runx2, TRAP (USCN), OPN (Ray Biotech), and RANKL (BioVendor Research Diagnostic) were measured using commercially available ELISA kit. Each measurement was performed according to the manufacturer's instructions. The intra-and interassay coefficients of variation were 2% and 7.9% respectively for MMP-9, <10% and <12% respectively for BMP-6, BGLAP, RANK, Runx2, TRAP, OPN, OPG, 2.6% and 6.3 % respectively for BMP-2, and 8.7 % and 9.15% respectively for RANKL.

*Leukotriene synthesis in myofibroblasts and monocytes (paper I)*
Monocytes were isolated from 6 healthy volunteers using standard procedure. Mononuclear leukocytes were resuspended in DMEM supplemented with 10% heat inactivated fetal bovine serum and antibiotics and left to adhere for 24 hours. Adherent monocytes were then harvested for RNA extraction and determination of 5-LO expression levels, as described above. In addition, myofibroblasts and monocytes were stimulated with the calcium ionophore A23187 (10 μM) and supernatants assayed for cysteinyl-LT concentrations by EIA.

*Ethical consideration*
The valvular project (**paper I, II, IV, V**) was approved by the local ethics committee (Reference number 2008/630-32), and all patients gave informed consent at the inclusion to the study. All experiments were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and were approved by the local ethics committee. All patients gave their informed consent prior to their inclusion in the study before performing carotid endarterectomy (Reference number 02/147; **paper III**).

# 4 RESULTS AND DISCUSSION

The corresponding papers are referred to with Roman numerals (I-V).

## 4.1 INFLAMMATION

### *4.1.1 Upregulation of the inflammation in aortic valve: the presence of the 5-lipoxygenase pathway*

As outlined in the introduction section calcified aortic valve stenosis is a progressive disorder, in which the initiating step is the active influence of local inflammation[8, 68] besides excessive remodeling of ECM directly connected to calcification[69, 187]. The complex interrelationship between these events leads to pronounced calcification, demonstrating elements of active bone formation, which is a final common pathway representing irreversible morphological changes within the diseased valve tissue. Additionally, the active impact of infiltrating immune cells, such as mast cells, macrophages and T-cells act as a trigger for valve calcification in an early vulnerable stage[68, 73], where eventually pharmacological intervention could retard/decrease the inflammatory burden. Previous landmark observations both in explanted human valve specimens[68] and in experimental animal models provided evidence for inflammation associated calcification paradigm using advanced molecular imaging technique[187], allowing to obtain a detailed histopathological picture of the early phase of the disease[80]. Based on those observations and the fact the activated infiltrating macrophages are the main source of the inflammatory mediators in aortic valve tissue, we have assumed that leukotrienes, a group of lipid mediators with potent proinflammatory properties, might have a pathogenetic role in the stenosis progression (**paper I**). Accordingly, the LT-pathway has been implemented in a variety of inflammatory conditions[91] and in cardiovascular pathologies[188].
To this end we have established a macroscopic dissection technique of human valve specimens, where we identified macroscopically normal, pliable, thickened and calcified regions in each individual leaflet, representing the entire disease spectrum from early signs to advanced stages (Figure 3), demonstrating the disease development in vivo. This model was subsequently used in **paper I, II, IV and V**.
Aortic valve specimens with different underlying aortic valve pathologies were included to the study, as it shown in the Table 1 (see in the Materials and Methods section).

### *4.1.2 qPCR-data analysis, obtained from stenotic aortic valves*

The local expression levels of the inflammation associated genes, such as CD 68, CD 8, 5-LO, IL-1β increased gradually as the valve progressed from normal through thickened to calcified part of the valvular tissue as shown in Figure 4. Since the mRNA levels derived from normal part of valves belonging to different valve pathologies (Table 1) did not show any significant differences, served as a control in all subsequent analysis. The results of macroscopic dissection technique used in studies: **paper I, II, IV, V** and were corroborated by previous histological classification with evidence of steady-state infiltration of inflammatory cells[68, 73]. In addition, the role of inflammation has been implicated as a critical link coupled to cuspal calcification through expression of MMPs[80], potent cysteine proteinases (cathepsines)[34, 74] leading to disruption/degradation of the valvular elastin content, which could serve as a *nidus* for hydroxyapatite-precipitation in addition to surrounding milieu favoring osteogenesis. Moreover, the transcript levels of IL-1β, expressed mostly by activated leukocytes, according to earlier observations[141] was abundantly present in the calcified valvular tissue, which was confirmed by our observation (Figure 4, **paper I**).

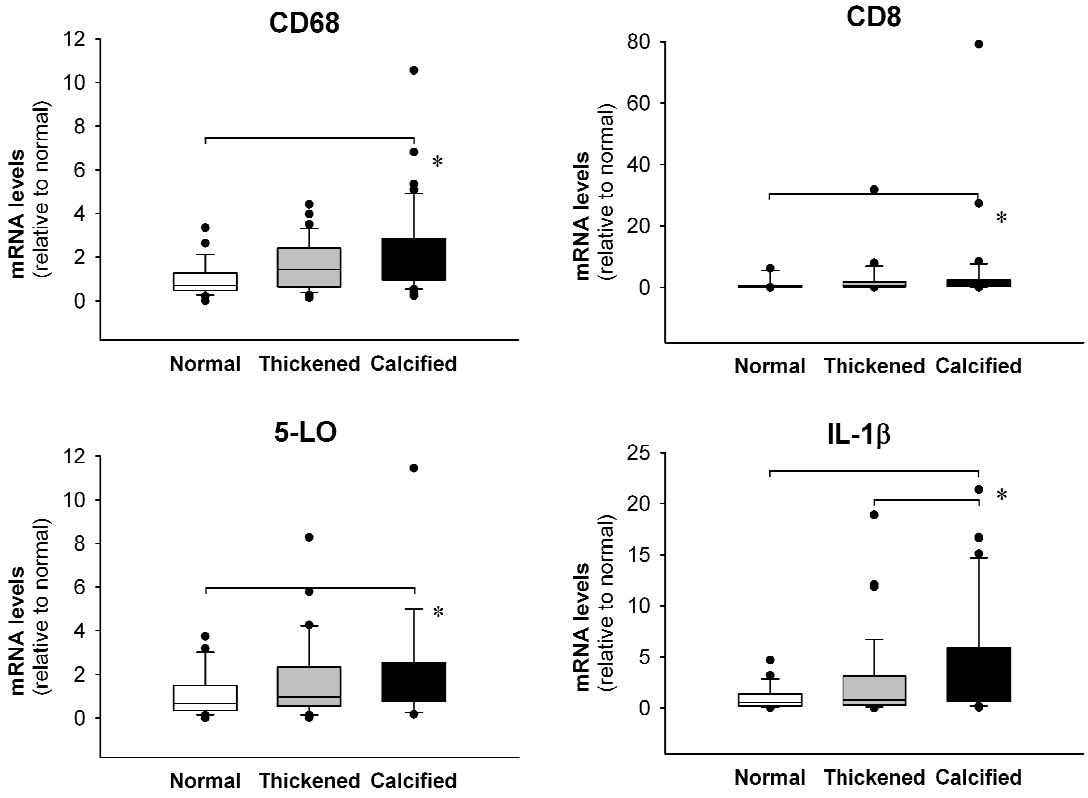



*Figure 4. Relative gene expression data of the inflammation associated genes: CD 68, CD 8, 5-LO, IL-1β (normalized to the normal area, derived from stenotic valves,* ***paper I****).*

Indeed, proinflammatory mediators, such as IL-1β, and T-cells via direct contact can activate macrophages and thus further enhance their local inflammatory activity, and the release of proteolytic enzymes[189, 190]. In parallel with atherosclerosis, as the stenosis progresses with increased inflammatory activity, $LTB_4$, one of the downstream effector of the 5-LO pathway acts as potent chemoattractant to further recruit more inflammatory cells to the inflamed valvular tissue and thus maintain LT-synthesis[92].

In **paper I** the transcriptional profile of CD 68 and 5-LO increased stepwise and demonstrated a positive significant correlation in all the three tissue classifications, providing evidence for that activated, infiltrating macrophages are the main source of local inflammatory activity, exerted in surgically removed human aortic valves. Whereas previous observations revealed evidence for the functional involvement of LT-pathway in human atherosclerosis[97, 191, 192], our recent publication (**paper I**) demonstrates for the first time the upregulation of the LT-cascade in explanted calcified human AV specimens. The lack of concomittant increase in the mRNA levels of the distal enzymes of the 5-LO pathway suggests the influence of other transcriptional regulatory mechanisms. Our data show a gradually increased with a preferential inflammatory activity observed in the calcified part, which might be connected to increased number of infiltrating immune cells and model the natural course of disease development.

### *4.1.3 The role of phenotypic plasticity of VICs, as a source of amplified inflammatory response exerted in aortic valve tissue*

VIC is the dominating cell type of aortic valve and responsible for the maintenance of valve integrity, competency and function in response to continuously changing metabolic, inflammatory and hemodynamic conditions. Phenotypic transdifferentiation of VICs, determined as an altered expression profile of cytoskeletal and surface proteins reflects an age-related adaptation mechanism of the valvular structure and is associated with a physiological maturation process[6]. However, altered phenotypic feature of VICs is also

implemented into the pathophysiology of stenosis progression. Under pathological conditions, a subset of interstitial cells may for example undergo phenotypic transdifferentiation toward bone-producing osteoblast-like cells with expression of osteogenic mediators, such as osteocalcin, bone sialoprotein, and osteonectin, which participate in the active calcification associated with AS[9,115]. One example of a possible epigenetically regulated pathway is the Runx2/Cbfa1, the master transcription factor involved into the osteoblastogenesis[62] can be inhibited by either by histone deacetylase activity (HDAC)[63], or by Notch, which is its transcriptional repressor. These facts suggested that the aortic valve's own structural cells may take active part in the valve's morphological changes with subsequent functional consequences. **Paper IV** was aimed to assess the underlying mechanism of one unexpected finding obtained in **paper I** demonstrating 5-LO activity in vimentin positive cells both in frozen sections of AV tissue and in primary culture of VICs (Figure 5). The hypothesis of **paper IV** was that epigenetic modification could lead to a transformation of VICs into leukotriene producing immune-like cells with a potential to aggravate the pathological processes. This was further corroborated in **paper I**, where conditioned media derived from different part of stenotic valves exhibited gradually increased CysLT concentrations (healthy: 28.7±6.2 pg/mL; thickened: 40.9±2.9 pg/mL; calcified: 47±2.3 pg/mL; $P<0.05$).

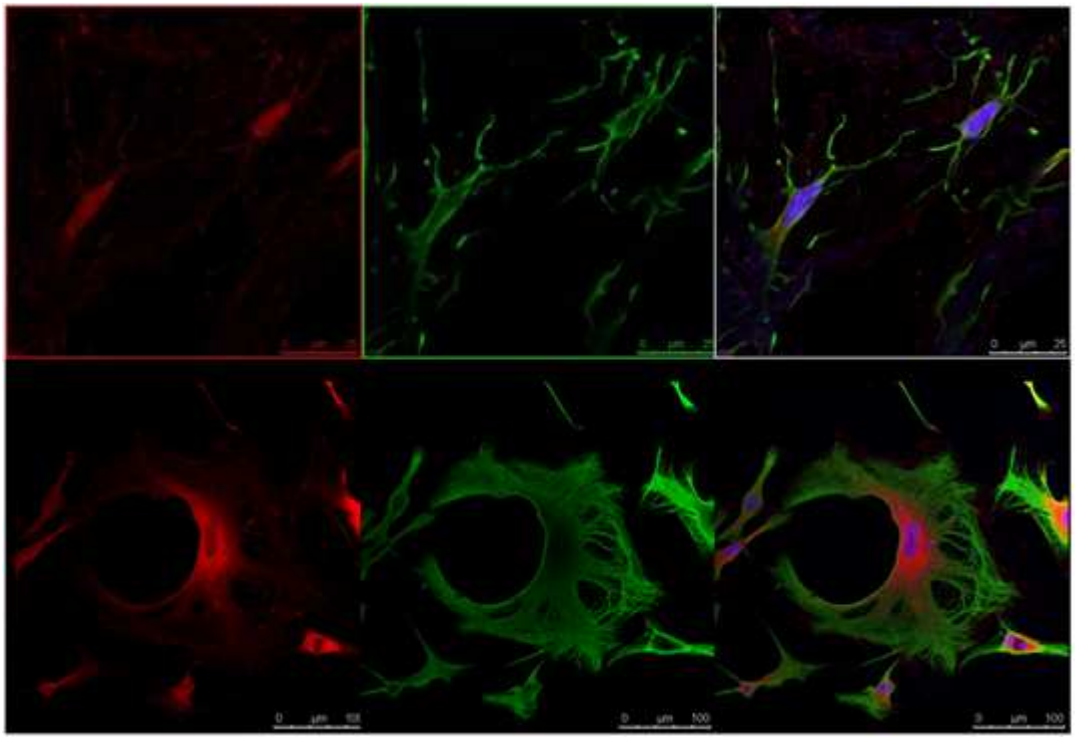


*Figure 5. Double Immunofluorescent staining showed co-localization of the 5-LO protein (red chromogen) with markers for VIC (vimentin, green chromogen), nuclei were counterstained with DAPI (merged, the last picture in each row). The upper row: frozen section from aortic valve (**paper I**); the lower row: primary culture of VICs (**paper IV**).*

Although, the mRNA levels of 5-LO were significantly higher in adherent monocytes (collected from healthy individuals; expressed as $2^{-\Delta CT}$: $3.2x10^{-3}\pm1.1x10^{-3}$ (n=6) versus $2.9x10^{-5}\pm4.3x10^{-6}$ (n=4), for monocytes and VICs, respectively; $P=0.01$) compared with VICs (**paper I**), suggesting that monocytes are more efficient producers of leukotrienes compared with VICs. However, the number of infiltrating immune cells even in the calcified part will still be lower compared it with those of VICs, which is the dominating cell type of the valve and serves as an additional source of inflammation in valvular tissue. According to previous observations, that the transcriptional activity of 5-LO is epigenetically regulated by promoter

methylation[193-195], the aim of **paper IV** was to assess the 5-LO promoter methylation status in AV tissue with its subsequent functional consequences to the transcriptional activity of 5-LO. Calcified part of valvular tissue showed a significantly lower amount of hypermethylated 5-LO (densely methylated) promoter compared to non-calcified part (Figure 6A). In addition, the alteration of the 5-LO methylation status was functional as demonstrated by the accompanied significant increase in 5-LO transcription (Figure 6B).

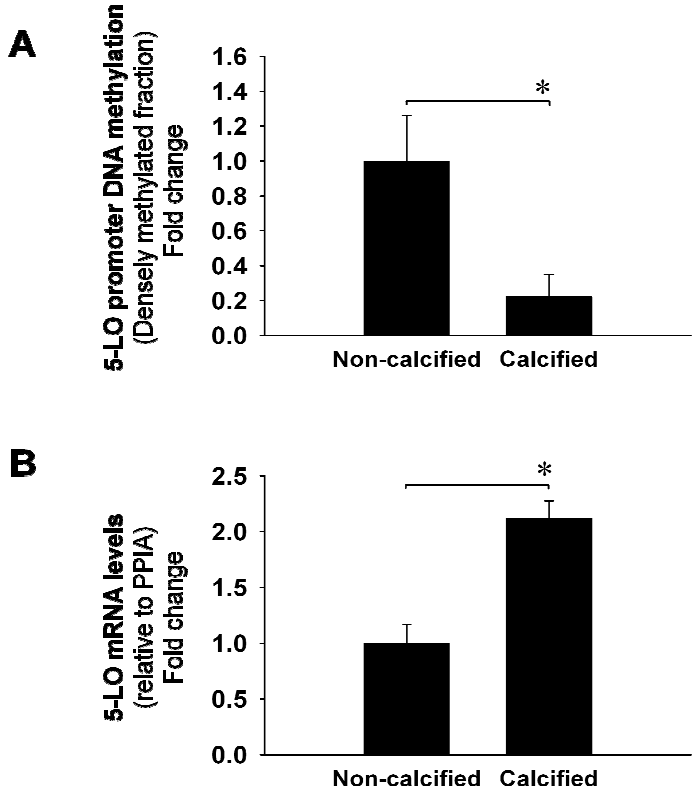



*Figure 6. Extent of methylation of 5-LO promoter region (A; n=8 preparations from 4 patients. The data are presented as a fold change compared to the methylation level in non-calcified tissue) and 5-LO mRNA levels (B; n=12 preparations from 6 patients) in non-calcified (thickened) and calcified parts of human stenotic aortic valves. *P<0.05; compared with non-calcified part.*

Additionally, there was a significant and inverse correlation between 5-LO promoter methylation and 5-LO mRNA levels in aortic valves (r=-0.5, p=0.04). These data point to the dynamic change of the methylation status of 5-LO promoter parallel with disease progression, which could further explain the enhanced inflammatory activity detected in end-stage valvular lesion. This is in accordance with earlier observation demonstrating still high inflammatory burden in end-stage aortic valve lesions[171]. However, it should be acknowledged, that interpretation of DNA methylation analysis data obtained from tissue samples is complicated by the fact that whole tissue contains heterogeneous cell populations with different methylation pattern. Therefore it cannot be excluded that a part of the observed decrease in the 5-LO methylation level is due to the infiltrating, less-methylated immune cells in the calcified part. As we demonstrated the 5-LO mRNA in VIC culture is several orders of magnitude lower than in leucocytes (**paper I**), cells are known for their abundant expression of 5-LO, probably related to their higher expression levels of downstream enzymes. However, the number of DNA copies derived from leukocytes will still be lower compared with VICs, and the methylation status analysis will mainly reflect the dominating cell type of the valve. To further confirm the phenotypic plasticity of VICs, *in vitro* cell culture experiments were performed on VICs, which has the advantage of studying uniform cell population. In this model, pharmacological inhibition of DNA methylation by using AdC, a

known hypomethylating agent increased 5-LO expression and decreased the degree of methylation in the CpG-rich region of the 5-LO promoter. This alteration of the 5-LO promoter methylation status was functional, as demonstrated by the increased $LTB_4$ production in the cell culture supernatants. It is interesting to note, that activated VICs are able to produce both $LTB_4$ (**paper IV**) and CysLTs (**paper I**), probably due to epigenetic alterations of the 5-LO promoter.
Collectively, our results are definite and provide cell biological findings on VICs phenotypic plasticity, which could serve as a functional link between epigenetics and the development and progression of aortic stenosis.

### *4.1.4 LT receptor expression in valvular tissue*

LTs act through activation of specific receptors. These receptors are expressed on inflammatory cells, for example neutrophils, macrophages, lymphocytes vascular and airway smooth muscle cells[92]. In **paper I** the transcriptional profile of $CysLT_1$ and $CysLT_2$ was unaltered throughout the valve, whereas the expression level of $BLT_1$ was significantly upregulated in thickened part, demonstrating presence of infiltrating leukocytes. Furthermore, the role of CysLT receptor signaling in structural cells is discussed in chapter: *4.1.6* within this discussion section (**paper III**).

### *4.1.5 The functional consequences of increased inflammatory activity to the stenosis severity*

After the demonstration the presence of the LT-pathway in valvular tissue, here it will be discussed its functional consequences. Earlier investigations established a potentially causal relationship between active influence of LTs and different cardiovascular pathologies, such as atherosclerosis[103, 196, 197], in stent restenosis after PCI[104], and abdominal aortic aneurysm[105]. As aortic valve stenosis is a slowly progressive disease with a disease continuum, beginning with early phases containing signs of enhanced inflammation, which is the key feature in early vulnerable phase, resembling to that existing in early atherosclerotic plaque formation, the hypothesis of **paper I** was that LT potentially could lead to hemodynamic progression. In order to assess the LT's effect on stenosis severity, correlation analyses were performed between qPCR data and echocardiographic parameters quantifying the stenosis severity. In the thickened part, representing the early vulnerable stage, the local expression level of several components of the LT-pathway, such as 5-LO, FLAP and $LTA_4H$ correlated inversely with the VTI-ratio (Table 2).

Table 2. Correlations between echocardiographic parameters and gene expression levels in thickened areas of stenotic aortic valves, including bicuspid and tricuspid valves.

| | | **5-LO** | **FLAP** | **$LTC_4S$** |
|---|---|---|---|---|
| **Age, y** | Correlation coeff. | 0.0147 | 0.142 | 0.0805 |
| | *P*-value | 0.931 | 0.406 | 0.653 |
| **AVA/BSA, $cm^2/m^2$** | Correlation coeff. | -0.309 | -0.303 | -0.245 |
| | *P*-value | 0.0663 | 0.0722 | 0.167 |
| **VTI-ratio** | Correlation coeff. | -0.351 | -0.385 | -0.302 |
| | *P*-value | **0.0357** | **0.0205** | 0.0869 |
| **P-mean, mm Hg** | Correlation coeff. | -0.021 | 0.101 | 0.352 |
| | *P*-value | 0.9 | 0.556 | **0.0446** |

In multiple linear stepwise regression FLAP remained significantly correlated with VTI-ratio (*P*=0.025). In addition, the transcript levels for $LTA_4H$, IL-1β, and MMP-9 correlated inversely with AVA/BSA (**paper I**). Moreover, the transcriptional profile of $LTC_4S$ demonstrated significant correlation with the P-mean (Table 2). Based on this classification[68], the early thickening of valve cusps before calcification represents an intermediate stage of aortic valve stenosis at which one can intervene and reverse the pathology. It is therefore interesting that our univariate correlation analysis in thickened areas of stenotic aortic valves revealed that, in addition to the already explored mediators of aortic stenosis, IL-1β[141] and MMP-9[198], [199], the mRNA levels for 5-LO, its activating protein FLAP, and the down-stream enzymes $LTC_4S$ and $LTA_4H$ correlated significantly with severity of stenosis. Collectively, upregulation of the LT-pathway in valvular tissue might have several effects through promoting inflammation and leading to stenosis progression. It was interesting to note, that the local expression level of all enzyme representing the entire LT-pathway correlated with the stenosis severity, whereas neither of CD 68 nor of CD 8 showed any significant correlation with the clinical stenosis severity. Correlations between several components of the LT-pathway, such as 5-LO, FLAP, $LTA_4H$, $LTC_4S$ and potent osteogenic morphogenes: BMP-2, BMP-6 and Runx2/Cbfa1 further confirmed the inflammation associated calcification, in which LTs might have a critical role (**paper I**).

### *4.1.6 CysLT's intracellular effects in human VICs and human SMCs*

Since our observation showed upregulation of inflammation in diseased aortic valve tissue, particularly the active influence of the LT-pathway which was correlated with the stenosis severity, we next elucidated the intracellular effects of one its candidate signal molecule (**paper I, III**). Because CysLT receptors were expressed on aortic valve's structural cells (Figure 7), we next examined the intracellular effects of one of its agonist acting as a potent inflammatory mediator: $LTC_4$. This was based on previously known fact, that aortic and airway wall express LT-receptors responding to CysLTs[92]. Because striking similarities exist between these two cell types (VIC and SMC) based on observations from experimental and clinical studies[200], the next step was to identify common pathways, critical for further functional understanding of the LT-cascade.

VIC is a mesenchymal cell type sharing many phenotypic similarities with vascular SMCs when it is not in an activated state[7]. Upon activation vascular SMCs and VICs exhibit phenotypical shift towards proliferating, secreting and migrating cells. In addition, earlier observations provided evidence for $CysLT_1$ mediated proliferation in primary culture of arterial SMC and their phenotypic alterations to a synthetic state in response to LT stimulation[19, 20]. Furthermore, earlier investigations provided evidence for LTs mediated $Ca^{2+}$-influx followed by activation of apoptosis[201].

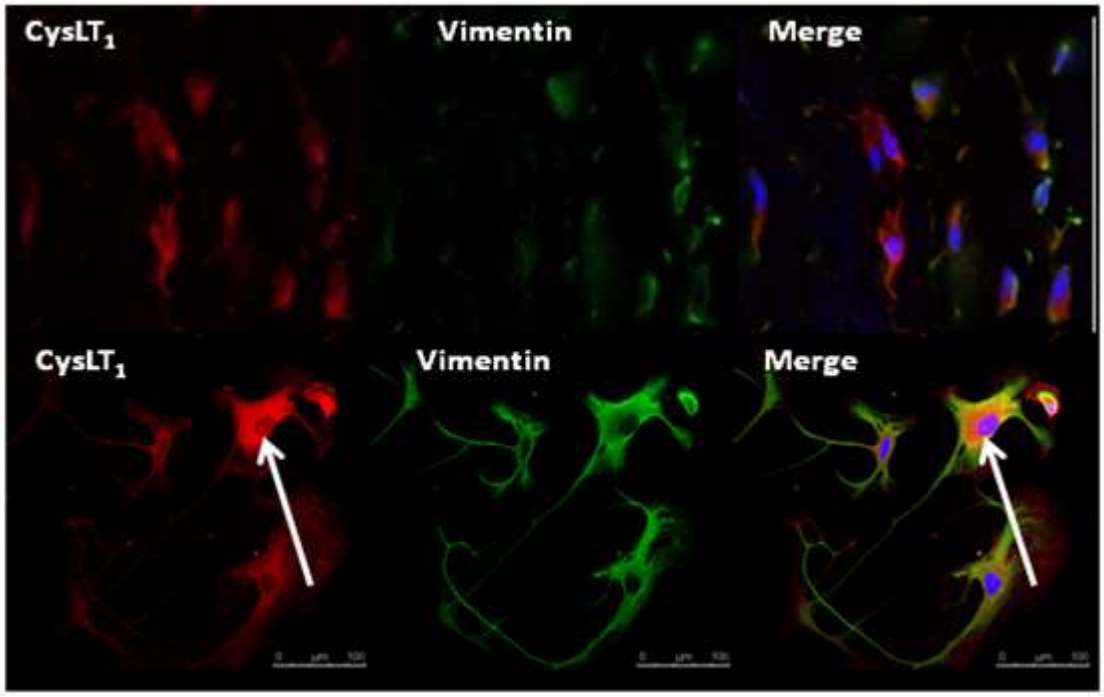



*Figure 7. Representative confocal photomicrographs of immunofluorescent labeling of human stenotic aortic valve (upper panel) and VICs (lower panel), respectively.*

To characterize the short, intermediate and long-term effects induced by $LTC_4$ on VICs different treatment durations were used.

Short-term/acute effect of $LTC_4$:
15 to 30 minutes stimulation by $LTC_4$ (1 nmol/L) caused significant rise in intracellular [$Ca^{2+}$] with the most pronounced increase detected in the nuclear and perinuclear region of the cell (Figure 8, left panel), indicating receptor translocation upon cell activation. The $CysLT_1$ receptor in some cases demonstrated nuclear inclusions, as indicated by white arrows in the lower panel of Figure 7. Similar expression pattern was detected in human coronary smooth muscle cells (**paper III**, Figure 11). These $LTC_4$-induced changes were dependent on EC $Ca^{2+}$-supplementation and were absent when $Ca^{2+}$-free cell culture medium was administered (Figure 8). Furthermore, these LT-induced changes were accompanied by time-dependent morphological alterations with an increase in the intracytoplasmatic vacuole formation, as shown in Figure 8, (left panel red arrows), dissipation of the mitochondrial membrane potential and increased ROS production, series of events leading to activation of cell death pathways (**paper I**). In addition, ROS production has previously been associated with calcification in diseased valve tissue[153] and also with osteoblast differentiation through induction of BMP-2[202]. Collectively, all those findings indicate that $LTC_4$ might act as a critical link between inflammation and *in situ* calcification in inflamed aortic valve tissue.

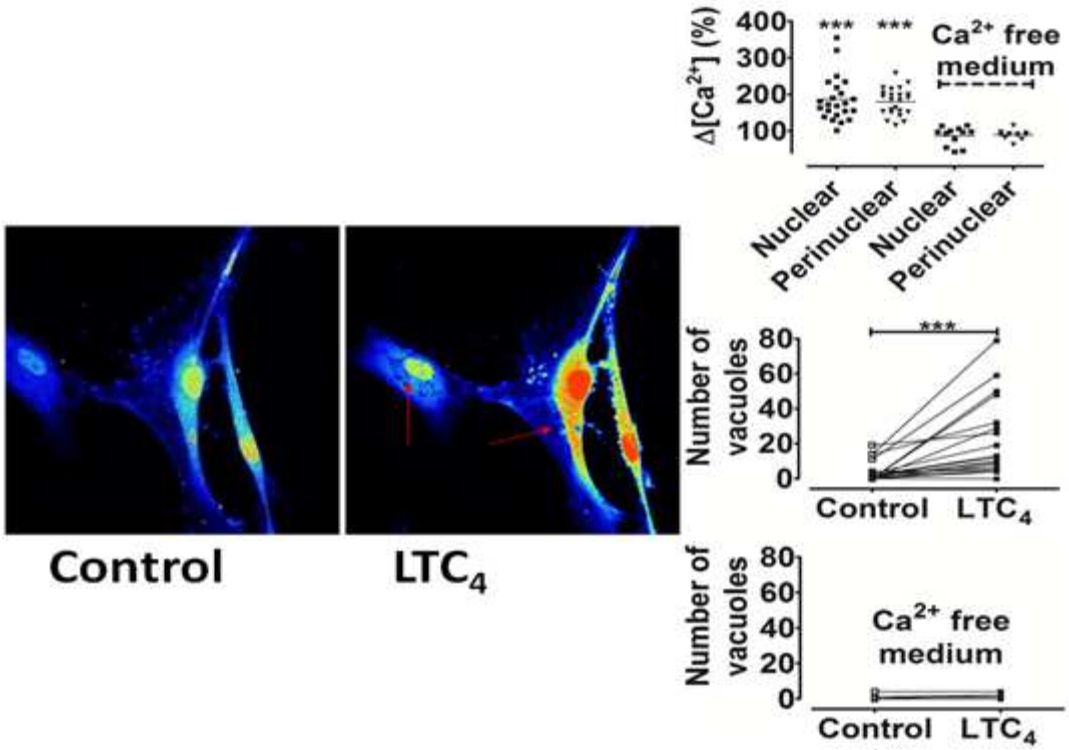



*Figure 8. $LTC_4$-mediated increase in nuclear and perinuclear $[Ca^{2+}]$. Representative confocal images of VICs loaded with the $Ca^{2+}$ indicator: Fluo-3. The left panel shows pictures before, indicated as "Control" and after treatment with $LTC_4$, indicated as "$LTC_4$", respectively. Red arrows indicate vacuole-formation after $LTC_4$ treatment. The right panel shows changes in $\Delta[Ca^{2+}]$ and intracellular vacuole formation in the presence or absence of EC calcium supplementation.*

Intermediate effect of $LTC_4$:

To further confirm $LTC_4$'s effect linking inflammation and cuspal calcification together 24 hours stimulation was used in primary culture of human VICs (**paper I**). As the Figure 9 shows the transcriptional level of BMP-2 and BMP-6 increased in a concentration-dependent manner and exhibited significant rise compared to the untreated cells.

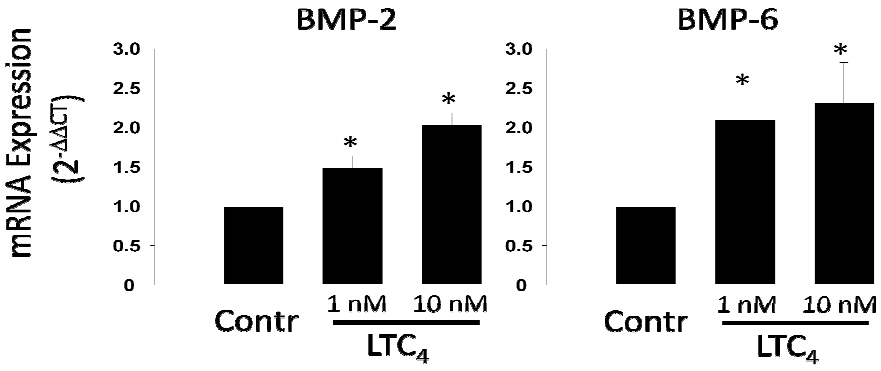



*Figure 9. mRNA levels for BMP-2 and BMP-6 in VICs after 24 hours exposure to $LTC_4$ (1-10nM), *P<0.05.*

Prolonged exposure of $LTC_4$ on VICs:
Sustained stimulation (two weeks) of primary culture of VICs with $LTC_4$ stimulated increased cell aggregation, calcified nodule formation, as evaluated by Alizarin red, a calcium specific staining (Figure 10)

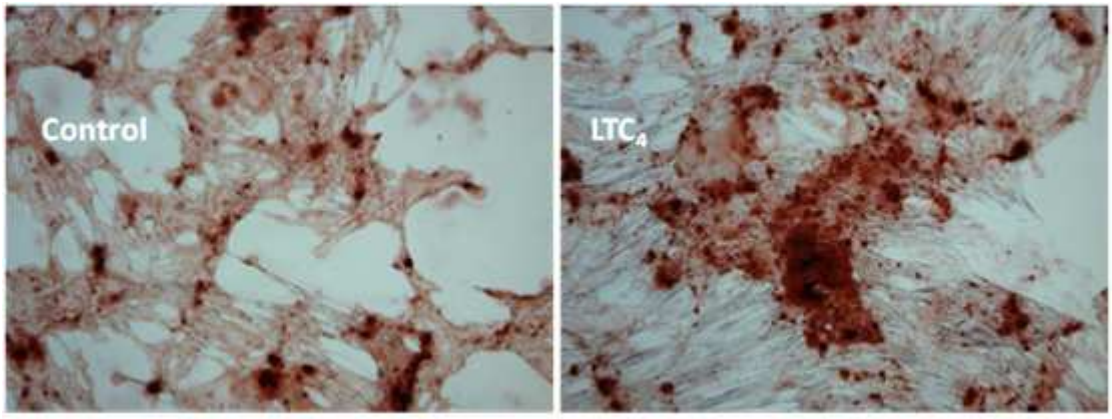



*Figure 10. Representative micrographs of Alizarin red stained human VICs cultured with β-glycerophosphate (10 mmol/L), $CaCl_2$ (1.5 mmol/L) and ascorbic acid (50 μg/mL) in the absence or presence of $LTC_4$ (100 nM) for 2 weeks.*

Taken together, all our findings are in line with previous observation on $CysLT_1$ receptor mediated eryptosis due to sustained intracellular calcium overload with subsequent deleterious effects on the cytoskeleton leading to cell scrambling and shrinkage[201]. In agreement with these $CysLT_1$ mediated observations earlier study demonstrated that $CysLT_1$ inhibitor, montelukast was able to reverse leukocyte apoptosis in chronic renal failure[203]. In addition, pharmacological inhibition of either the multidrug resistant protein 1 (MRP1) by MK 571, which promotes the transport of $LTC_4$ from the intra- to the extracellular compartment, or the $CysLT_1$ receptor by montelukast, reduced vascular ROS production and improved endothelial function[204]. Based on our experimental observations on $LTC_4$-induced nuclear calcium signaling and nuclear translocation of its receptor, the next study (**paper III**) was aimed to further characterize and extend these findings to vascular SMCs derived from human coronary arteries. Previous observations reported three-fold higher expression level of $CysLT_1$ compared to $CysLT_2$ in atherosclerotic human carotid arteries[94] with increased contractile response evoked by CysLTs[21]. In contrast, under physiological conditions systemic vessels are unresponsive to CysLT stimulation[22], which further indirectly corroborates the increased vascular sensitivity to LTs in diseased arteries mediated by $CysLT_1$ receptor[94]. The hypothesis of this study was that inflammatory environment could lead to upregulation of $CysLT_1$ receptor with subsequent functional consequences on gene expression profile leading to loss of intact vascular sensitivity towards atherogenic properties (**paper III**).
In accordance with the findings obtained in VICs reported in **paper I**, double fluorescence immunohistochemistry showed $CysLT_1$ protein expression in α-smooth muscle actin positive cells both in human specimen derived from atherosclerotic lesion and in cell culture of human coronary SMCs (Figure 11) demonstrating perinuclear localization pattern. Furthermore, LPS, IL-6 and IFN-γ stimulation induced increased $CysLT_1$ receptor expression in a time dependent manner (Figure 12). To evaluate whether $CysLT_1$ receptors expressed on vascular SMC were functional, calcium changes in human coronary artery SMC were studied using the fluorescent calcium indicator Fluo-3.

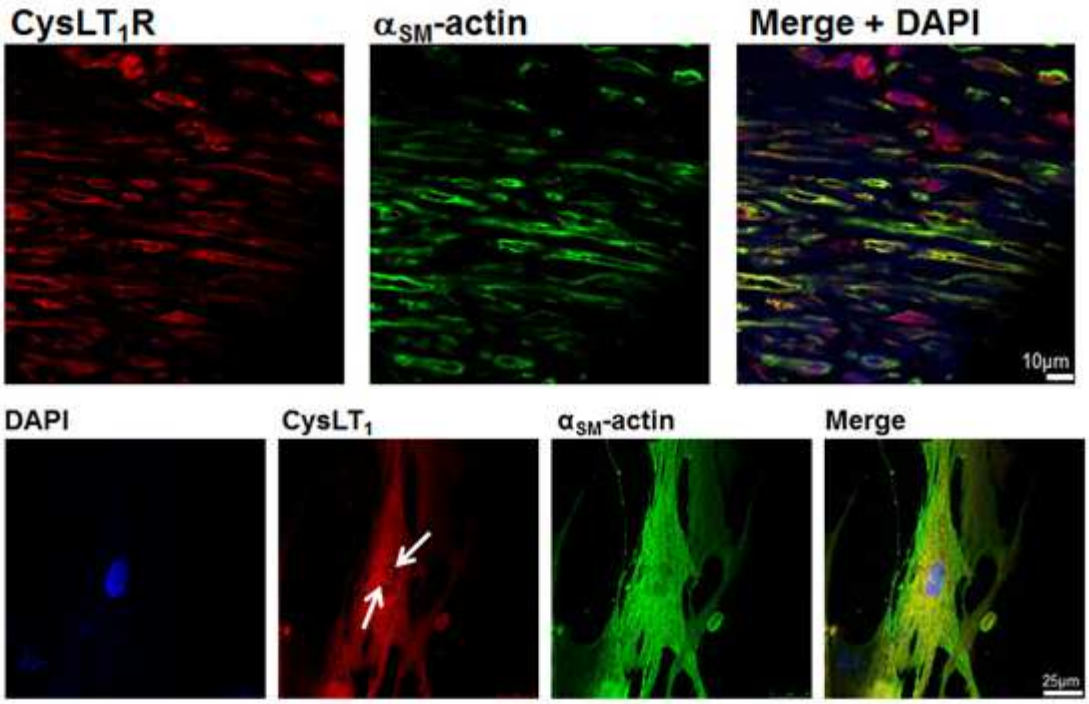



*Figure 11. $CysLT_1$ expression in α-smooth muscle actin positive cells in atherosclerotic lesion from human carotid artery (upper panel) and in cell culture of human coronary SMC (lower panel).White arrows indicate nuclear inclusions.*

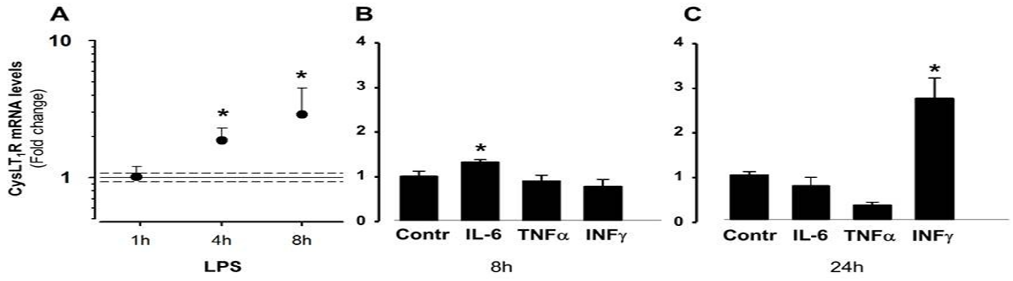



*Figure 12. $CysLT_1$ receptor expression in human coronary artery SMCs is upregulated by pro-inflammatory stimuli. Real-time quantitative TaqMan RT-PCR for $CysLT_1$ receptor mRNA in SMCs incubated in the absence and presence of LPS (10 μg/ml) for 1, 4, and 8 hours (A) and IL-6 (20 ng/mL), TNF-α (10 ng/mL), or IFN-γ (20 ng/mL) for either 8 hours (B) or 24 hours (C). Results are expressed as fold increase compared with untreated cells (n=3–5). *P<0.05 vs. time-matched control.*

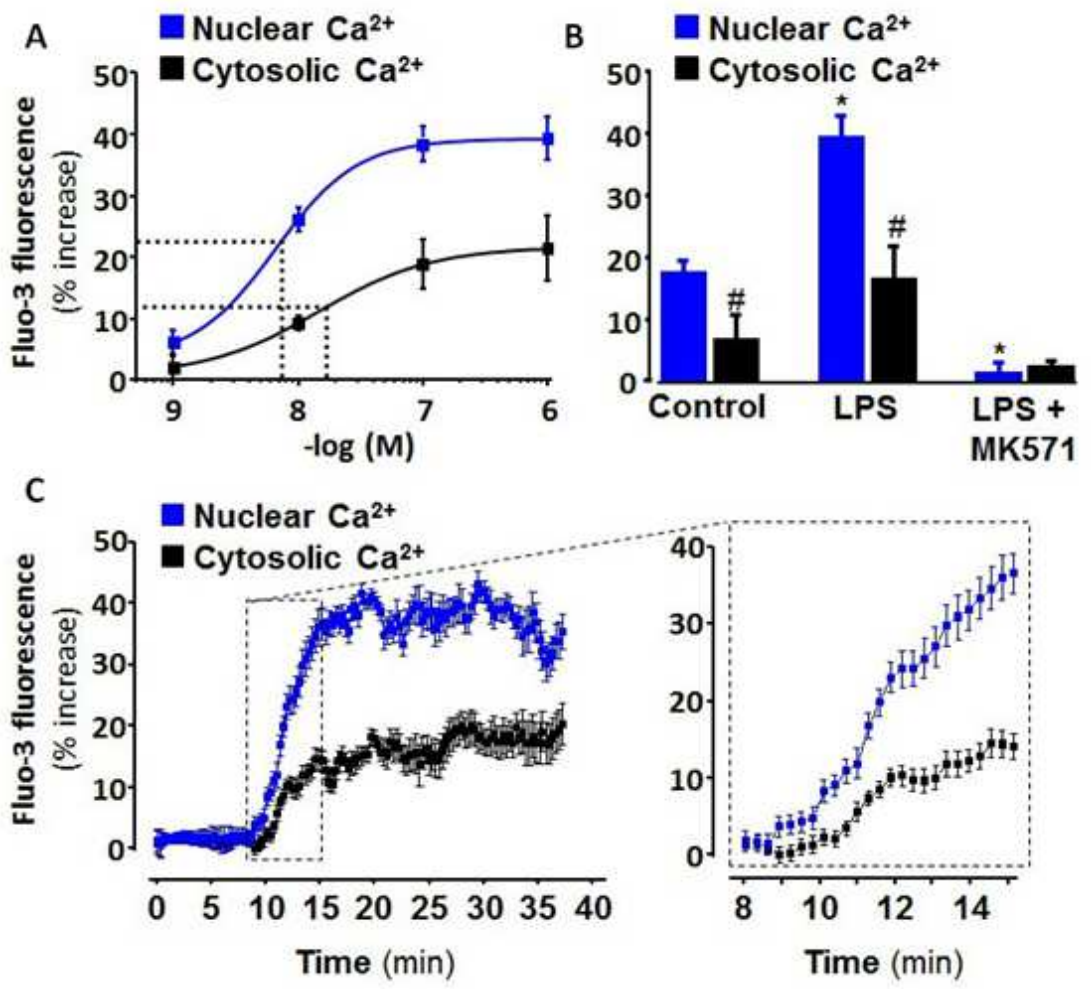



*Figure 13. A: concentration-response curves for $Ca^{2+}$ fluorescence in nuclei and cytosol of SMCs incubated for 48 hours in the presence of LPS (10 μg/ml). B: $Ca^{2+}$ fluorescence in nuclei and cytosol of SMCs incubated for 48 hours in the absence (Control) or presence of LPS (10 μg/ml) prior to stimulation with $LTC_4$ (1 μM, 30 min). C: The time course of the $LTC_4$-induced calcium increase shows that the increase in nuclear calcium (Blue symbols) preceded the increase in cytosolic calcium (Black symbols).*P<0.05 vs. controls, #P<0.05 N vs C in each condition.*

$LTC_4$ induced a dose-dependent increase in intracellular calcium was observed, which was predominantly located in the nucleus (Figure 13 A). The $LTC_4$-induced calcium increase was significantly higher in LPS-pretreated cells compared with untreated cells (Figure 13 B). In LPS-pretreated cells, the $LTC_4$-incuced increase in nuclear calcium was significantly inhibited by the $CysLT_1$ receptor antagonist MK571 (Figure 13 B). The time course of the $LTC_4$-induced calcium increase in the nuclear and cytosolic compartments is shown in Figure 13 C. The increase in nuclear calcium preceded the increase in cytosolic calcium. Taken together, priming of vascular SMCs with LPS and other inflammatory cytokines up-regulated $CysLT_1$ receptor mRNA and enhanced $LTC_4$-induced effects, similar to that observed in endothelial cells[205]. Intracellular calcium signaling in nucleated cells might have dual properties depending on the kinetics of the cytosolic and nuclear calcium oscillator generating periodic pulses of calcium. While periodic calcium oscillations in the cytoplasmatic compartment are physiological[206], sustained intracellular calcium overload could stimulate activation of apoptotic pathways[207]. In our cell culture of human SMCs, $LTC_4$ stimulation resulted in increase in calcium both in the nuclear and the cytoplasmatic compartments of the cell with a sustained increase reaching a plateau level without any further significant changes in calcium concentrations (Figure 13 C). Collectively, this type of changes in intracellular calcium concentration pattern could be connected to apoptosis.

The next steps within this project was to assess the downstream effectors in response to enhanced nuclear calcium signaling, determined as altered phenotypic feature of vascular SMCs with synthetic potential[19]. Microarray experiment revealed SERPIN B2/PAI-2 as one of the most significantly upregulated genes, which was confirmed by TaqMan real time PCR (Figure 14 A) and at the protein level, using ELISA technique (Figure 14 B) in the supernatants derived from $LTC_4$ stimulated human coronary SMCs.

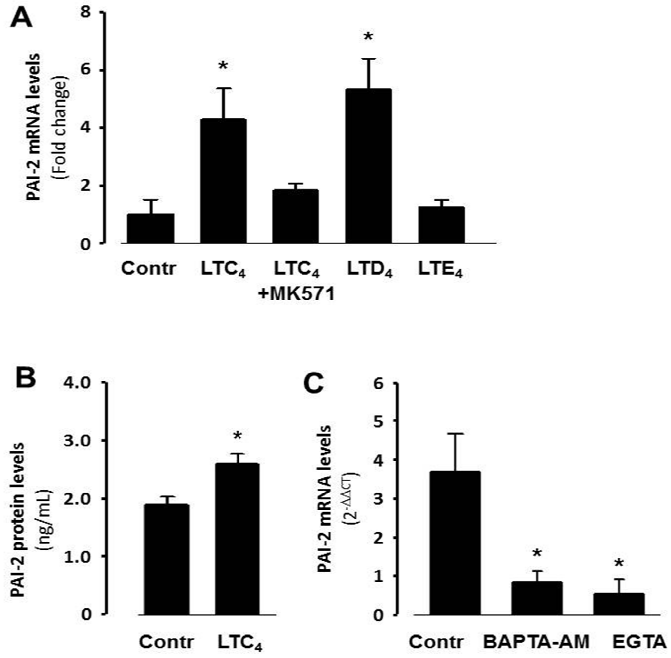



*Figure 14. $LTC_4$-induced upregulation of PAI-2 in human coronary artery SMC. A: Real time qPCR for PAI-2 mRNA in human coronary artery SMC incubated in the absence and presence of $LTC_4$, $LTD_4$ or $LTE_4$ (1 μM) for 24 hours. In some experiments, cells were pretreated with the $CysLT_1$ receptor antagonist MK571 (1 μM) for 1 hour before addition of $LTC_4$. *P<0.05 vs. non-$LTC_4$ stimulated Contr (n=3–6). B: PAI-2 concentrations in supernatants from human coronary artery SMC incubated 24 hours in the absence (Contr) or presence of $LTC_4$ (1 μM). *P<0.05 vs. Contr (n=7). C: Increase in PAI-2 mRNA levels induced by $LTC_4$ (1 μM) in human coronary artery SMC incubated in the absence (Contr) or presence of either BAPTA-AM or EGTA. *P<0.05.*

In addition, these changes in gene expression profile of PAI-2 were significantly abolished in the presence of BAPTA-AM and EGTA, known calcium chelators (Figure 14 C).
PAI-2 belongs to the serine proteinase inhibitor family preferentially expressed in unstable carotid plaque[208]. In addition, PAI-2 but not PAI-1 is relatively resistant to oxidative conditions[209] which characterizes inflammatory states and thus can be used to monitor sustained inflammatory activity. In addition, other studies demonstrated upregulated PAI-2 expression levels in vascular SMCs treated by TNF-α[210], or by conditioned media derived from macrophages activated by oxidized LDL[211]. Furthermore, the presence of PAI-2 within the extracellular space of the atherosclerotic vessel wall may contribute to control wound healing process after plaque rupture[208].
Although the microarray analysis did not reveal any alterations in PAI-1 gene expression profile, which is the main inhibitory SERPIN, but according to a previous finding[212] PAI-1 promoter region contains several responsive sites for cAMP response element binding protein (CREB) as well. Ingenuity Pathway Analysis identified several functional gene networks

predicted to be significantly affected by $LTC_4$ stimulation. One of the most interesting finding was the involvement of CREB, a transcription factor and member of the leucine zipper family of DNA binding proteins, which is known to be activated by nuclear calcium[213]. Another pathway connected to $LTC_4$ induced gene expression was the receptor activator of nuclear-κB (NF- κB), which previously has been shown to be activated followed by $CysLT_1$ ligation[94]. Collectively, observations in **paper III** confirmed and extended our previous findings reported in **paper I** demonstrating perinuclear $CysLT_1$ expression in response to inflammatory stimuli. In addition, $LTC_4$ induced increase in nuclear calcium signaling was associated with alteration of gene expression profile, demonstrating proatherogenic properties.

## 4.2 THE ROLE OF NITROSATIVE STRESS--CONNECTION TO THE CELL DEATH PATHWAYS; DIFFERENCES BETWEEN TAV AND BAV

Previous observations suggested that oxidative stress may contribute to the hemodynamic progression of AS[117]. *In vitro* experiment of porcine VICs demonstrated that NO donors attenuate osteogenic differentiation[214], which suggests that the presence of oxidative stress might be connected to hemodynamic progression of AS. Since we found in **paper I**, that leukotriene signaling was associated with an increase in ROS formation in valvular interstitial cells, the **paper II** was initiated with the aim to explore the nuclear enzyme PARP-1 expression, as a downstream effector of oxidative stress in the context of AS. The transcriptional profile and the topological distribution of PARP-1 in human AV, and its relation to the severity of stenosis in different valve anatomies including both tricuspid and bicuspid valves were investigated. Using the macroscopic dissection technique establised and validated in **paper I**, surgically explanted human stenotic aortic valves were used for macroscopic dissection followed by Taqman qPCR for PARP-1. In addition, cell culture studies were performed to assess the relationship between leukotrienes and PARP-1. Our results demonstrate that the thickened areas of stenotic valves with tricuspid morphology expressed significantly higher levels of PARP-1 mRNA compared with the corresponding part of bicuspid valves (Figure 15). These findings provide a first suggestion of potential differences in DNA damage pathways between bi- and tricuspid valves. Furthermore, the quantitative gene expression levels of PARP-1 correlated with aortic stenosis severity in tricuspid aortic valves, whereas no correlations were observed in bicuspid valves (Table 3).

Table 3. Correlations between echocardiographic parameters and gene expression levels for PARP-1 in thickened areas of stenotic aortic valves (**paper II**)

| | | **PARP-1 in TAV** | **PARP-1 in BAV** |
|---|---|---|---|
| **AVA, cm²** | Correlation coeff. | -0.46 | 0.174 |
| | *P*-value | **0.0469** | 0.542 |
| **AVA/BSA, cm²/m²** | Correlation coeff. | -0.498 | -0.0462 |
| | *P*-value | **0.0296** | 0.868 |

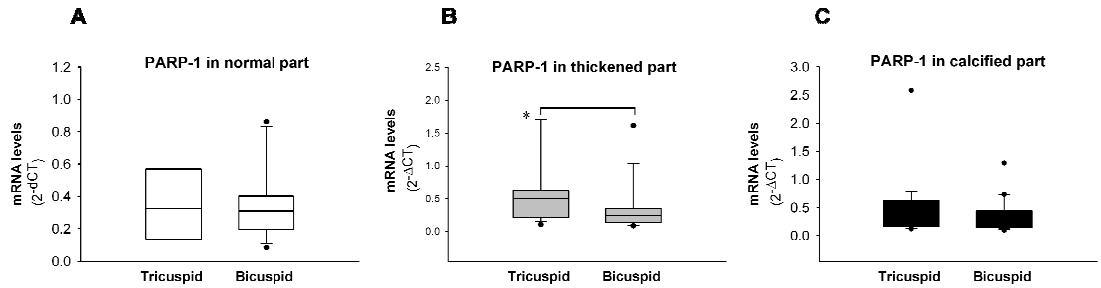



*Figure 15. qPCR data in TAV and BAV in normal (A), thickened (B) and calcified (C) part of stenotic valves. (**paper II**)*

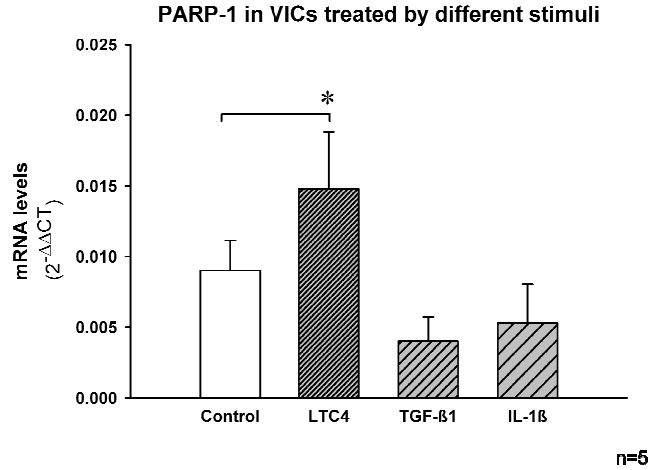



*Figure 16. In vitro cell culture experiment on VICs using $LTC_4$, TGF-β1, and IL-1β. Bars represent mean±SEM. The transcript level of PARP-1 exhibited significant increase at 1nM $LTC_4$ (P=0.006). (**paper II**)*

*In vitro* cell culture experiment showed that $LTC_4$ lead to significantly elevated mRNA levels of PARP-1 in VICs (Figure 16). These effects were observed at low concentrations of $LTC_4$, which is in line with our previous study, indicating $LTC_4$ as a potent inducer of PARP-1 in VICs. Along with previously demonstrated intracellular effects of $LTC_4$ in VICs (**paper I**) and in vascular SMCs (**paper III**) containing increased nuclear calcium, ROS production, dissipation of the mitochondrial membrane potential, increased sensitivity for $CysLT_1$ receptor with subsequent altered gene expression profile and as an effect of prolonged stimulation, increased calcium nodule formation, collectively all these events link inflammation through activation of cell death pathways and cuspal calcification together. The activation of PARP-1 pathway, as a result of increased ROS production in response to $LTC_4$ stimulation potentially could serve as an effector to mediate $LTC_4$'s intracellular effects.

Previously has been shown, that PARP-1 regulates the intracellular trafficking of several transcription factors and thus modulates the cellular response to oxidative injury[215], for example for NF-κB. In addition, we have demonstrated the activation of NF-κB in $LTC_4$ primed vascular SMCs (**paper III**), which might be mediated through ROS-induced PARP-1 activation.

Some substantial differences exist between TAV and BAV, not only regarding the different etiology, but also the natural course of the disease. We previously demonstrated that TAV represents a more pronounced inflammatory state compared with BAV (**paper I**), as the transcript levels of CD 68 and CD 8 were significantly higher in TAV. In addition, the hemodynamic progression of the stenosis is accelerated in BAV compared with TAV, probably due to altered systolic flow distribution[56].

In conclusion, in human aortic stenosis, PARP-1 expression levels were higher in tricuspid compared with bicuspid valves, and were correlated with stenosis severity only in tricuspid valves. Since PARP-1was induced by leukotrienes, these data suggest that oxidative stress

and valvular DNA-damage through PARP-1 activation may be associated with inflammation and the stenosis severity in tricuspid aortic valves.

## 4.3 THE ROLE OF BONE TURNOVER, VALVULAR OSTEOCLASTS AND THEIR RELATION TO THE CLINICAL STENOSIS SEVERITY

Based on inflammation-dependent calcification paradigm[80, 145] and on our observation on $LTC_4$-induced calcification (**paper I**) we next characterized the calcification process through assessment of the local transcriptional profile of genes with osteoinductive and osteoresorptive potential and their association with the clinical stenosis severity (**paper V**).

*In vivo* experimental data provided evidence for opposing effects of inflammation in the vasculature, aortic valve and in skeletal bone tissue[151]. Whereas inflammation is associated with increased calcification in arterial wall and aortic valve, the mineral content of inflamed bone decreases, revealing paradox simultaneous osteolysis[151]. Longitudinal epidemiological studies in patient cohorts with osteoporosis demonstrated high prevalence of cardiovascular disease suggesting common promoting factor for osteoporosis and co-existing vascular and valvular disease[216]. This raises an important question, if osteoclasts with their known resorptive function can be used in the context of AS to remove mineral content and thus limit the progression rate. The method using local injection of osteoclasts or using RANKL to recruit osteoclasts to efficiently resorb calcification, as a potential biological treatment of calcified tendinitis has been proposed[217]. However, the role of vascular and valvular osteoclasts has not been investigated in the context of AS progression.

All previous studies focused on the role of osteoblasts in AS and there is little conclusive information about the influence of osteoclasts to the calcification process. Mohler et al. were the first to describe osteoclasts in heavily calcified human aortic valve tissue[70]. According to the facts obtained in skeletal bone tissue, the bone remodeling in calcified aortic valves has its origin in microfractures at multiple sites of the valve, at which osteoclasts and osteoblast recruited, together building up a complex system[152].

The aim of **paper V** was to assess circulating mediators of bone homeostasis and their association to the severity of stenosis and to explore the spatio-temporal distribution of bone turnover in different parts of calcified aortic valve tissue, based on dissection technique reported in **paper I**. As we earlier described (**paper I, II**) human aortic valves were used for RNA extraction followed by qPCR.

Osteoclasts are formed by fusion of monocytes/macrophages, confirming their hematopoietic origin. Recent published data described osteoblast-osteoclast cross talk[136], demonstrating regulatory effect of osteoblasts on the recruitment and activity of osteoclasts through expression of RANKL which can be abolished by OPG, a member of TNF receptor superfamily, taking active part of the regulation of bone turnover[218]. Once RANKL binds to its receptor (RANK) on the osteoclasts a series of events activated stimulating bone resorption, ECM degradation though activity of cysteine proteases, MMP and osteopontin phosphatase, also known as TRAP, a generator of reactive oxygen species[219]. In advanced stages of AS parallel to mature lamellar bone formation, diseased valves are places of intense remodeling of the ECM[69] leading to hemodynamic progression of stenosis severity. Our previous observation showed significantly upregulated transcript levels of MMP-9 and its correlation to the stenosis severity confirms the above notion[142] (**paper I**).

Table 4. Patients' characteristics (**paper V**)

| | **Aortic valve stenosis** | | | |
|---|---|---|---|---|
| | **All patients** | **Bicuspid aortic valve** | **Tricuspid aortic valve** | **Overall *P*-value** |
| **No. of patients** | 46 | 23 | 23 | |
| **Male, n (%)** | 29 (63%) | 16 (35%) | 13 (28%) | |
| **Age, y** | 70.9 (28.5-87.1) | 64.4 (28.5-82.4) * | 76.2 (57.9-87.1) | **<0.001** |
| **BMI, kg/m²** | 26.9 (19.2-37.42) | 26.9 (19.2-34.7) | 26.3 (21.6-37.4) | 0.682 |
| **Creatinine** | 78.5 (47-156) | 76 (47-134) | 79 (60-156) | 0.235 |
| **HbA1c** | 4.5 (3-6.3) | 4.5 (3.9-6.3) | 4.5 (3-6.3) | 0.659 |
| **t-cholesterol (mmol/L)** | 4.85 (2.7-6.7) | 5 (2.7-6.7) | 4.7 (3.3-6.1) | 0.094 |
| **LDL-C (mmol/L)** | 2.9 (1.3-4.4) | 3.05 (1.3-4.4) | 1.8 (1.4-4.2) | 0.26 |
| **HDL-C (mmol/L)** | 1.4 (0.5-2.9) | 1.4 (0.5-2.9) | 1.4 (0.6-2.5) | 0.785 |
| **Triglyceride (mmol/L)** | 0.99 (0.38-13.8) | 0.91 (0.42-13.8) | 1.1 (0.38-3.7) | 0.775 |
| **Hs CRP** | 1.35 (0.2-18) | 1 (0.2-18) | 1.5 (0.35-14.6) | 0.59 |
| **AVA, cm²** | 0.73 (0.45-1.2) | 0.76 (0.47-1.2) | 0.72 (0.45-1) | 0.134 |
| **AVA/BSA, cm²/ m²** | 0.39 (0.26-0.6) | 0.4 (0.3-0.6) | 0.38 (0.25-0.58) | 0.255 |
| **VTI-ratio** | 0.195 (0.14-0.37) | 0.2 (0.16-0.28) | 0.2 (0.14-0.37) | 0.613 |
| **Aorta Vmax, m/s** | 4.475 (3.36-5.8) | 4.5 (3.4-5.7) | 4.5 (4-5.8) | 0.255 |
| **P-mean, mm Hg** | 50 (27-93) | 51 (27-85) | 49 (37-93) | 0.403 |
| **Left ventricular EF, %** | 61.9 (42-78) | 61% (44-78) | 64%(42-75) | 0.55 |
| **LVEDD, mm** | 47 (36-63) | 49 (36-63) | 47 (37-57) | 0.234 |
| **Overall calcium score** | 4 (3-5) | 4 (4-5) | 4 (3-5) | 0.54 |

In **paper V** only patients with AS with either bi-or tricuspid valve anatomy were involved as shown in the Table 4. Among the clinical and echocardiographic parameters it was only the age which differed significantly between the groups. Plasma levels of OPG were significantly higher in BAV patients (**paper V**), whereas other circulating biomarkers were not significantly different between the groups.

Given that the measured biomarkers indicated an association of aortic stenosis severity with the osteoclast-derived TRAP (as discussed in this chapter); we assessed the presence of osteoclasts in human aortic valves. Hematoxylin-eosin and immunofluorescence staining of calcified part revealed multinucleated cells in areas adjacent to active bone formation (Figure 17).

In parallel to atherosclerosis, where several proteins - for example those taking part in ECM degradation and thus contributing to plaque destabilization - circulating plasma levels were connected to risk factor status both in a population without history of coronary artery disease (CAD)[220] and in patients with known CAD[221] or to clinical outcome[222, 223], we next evaluated the circulating plasma levels of proteins related to osteoblast and osteoclast activities.

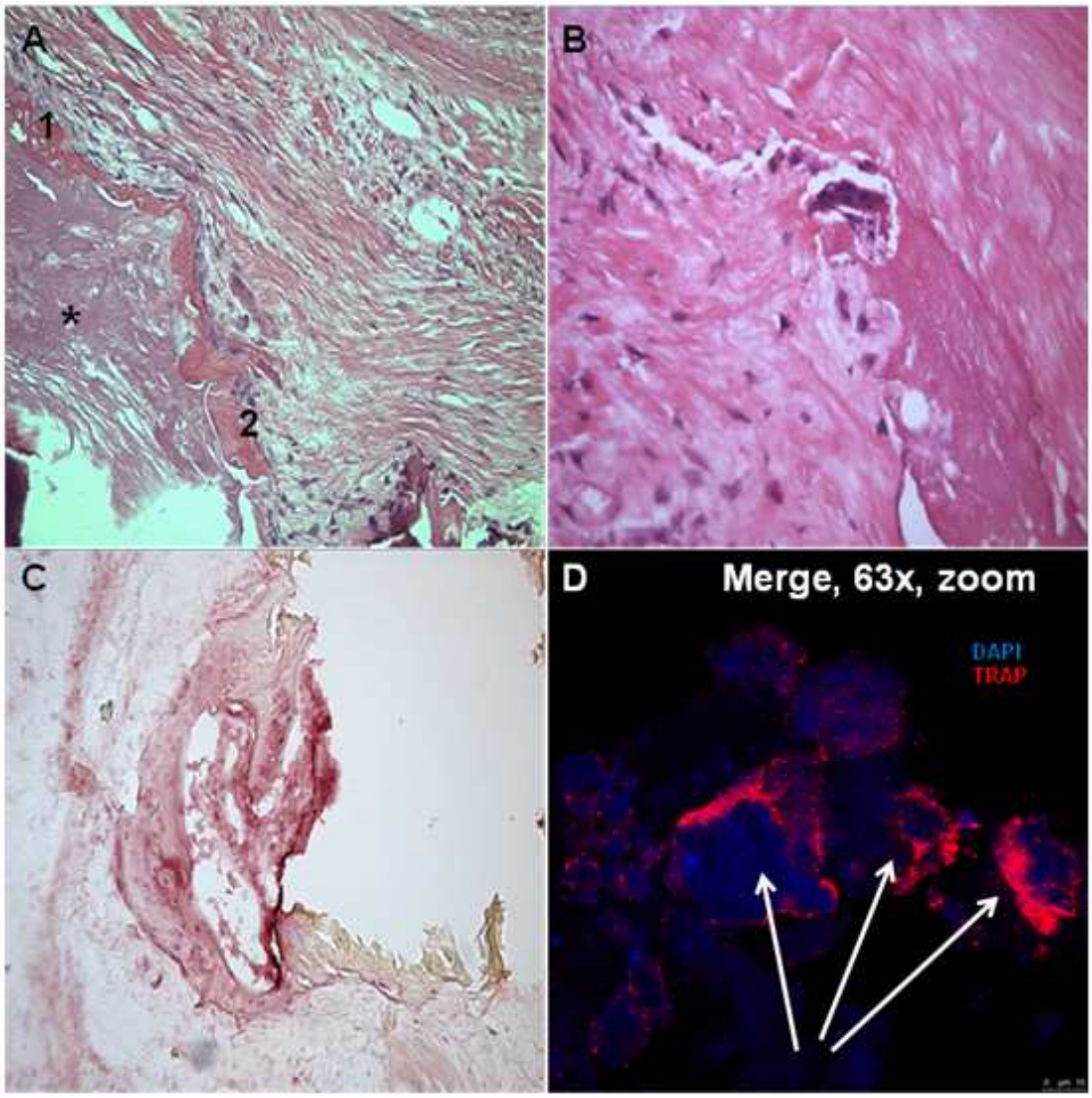



*Figure 17. A: Overview of a calcified part of aortic valve demonstrating 1: cartilaginous nodule in close vicinity to atheromatous plaque containing dystrophic calcification, indicated as an *; 2: mature lamellar bone with active osteoclastic resorption, original magnification 20x. B: osteoclast sitting in a Howship`s lacuna/resorption space within a calcified part of aortic valve. Hematoxylin and eosin, original magnification 40x; C: light microscopy of TRAP activity staining identifies an area with strong TRAP activity in close vicinity to calcification, original magnification 10x. D: immunofluorescent staining for TRAP counterstained with DAPI demonstrates multinucleated cells, as arrows indicate.*

The results of the age-, gender- and aortic valve anatomy adjusted multivariable regression model are shown in Table 5, demonstrating β-coefficients. In this model, the plasma concentrations of TRAP, Runx2/Cbfa1 and RANKL were significantly correlated with P-max, P-mean, VTI-ratio, respectively. In addition, ratios of RANKL/RANK and RANKL/OPG were significantly related to AVA/BSA and VTI-ratio. The plasma level for BMP-2 was significantly correlated with overall calcium score (Table 5).

These observations were confirmed at the tissue level by the enhanced local transcriptional profile of genes taking part in osteoclast differentiation, activity and survival, detected in calcified valvular tissue (Figure 18). Although the expression level of the master transcription factor for terminal osteoblast differentiation Runx2/Cbfa1 was unaltered throughout the valve, circulating (Table 5) and local Runx2/Cbfa1 expression levels exhibited significant correlation with the stenosis severity (β-coefficient=-0.027; *P*=0.03).

In addition, mRNA levels for TRAP in the normal part demonstrated inverse correlation with AVA and AVA/BSA (β-coefficient=-0.03, *P*=0.02; β-coefficient=-0.05, *P*=0.03, respectively), and the transcriptional profile of MMP-9 was also significantly correlated with

the stenosis severity as we reported in **paper I**. Taken together, these findings suggest a correlation between bone turnover including osteoblast differentiation and markers with osteoresorptive function with stenosis severity both at the systemic and tissue level.

Table 5. Multivariable regression, adjusted for age, gender and aortic valve anatomy for log-transformed circulating biomarkers*P<0.05; **P<0.01.

| | **Runx2/Cbfa1** | **BMP-2** | **TRAP** | **MMP-9** | **RANKL** | **RANK** | **RANKL/OPG** | **RANKL/RANK** |
|---|---|---|---|---|---|---|---|---|
| **AVA/BSA, $cm^2/m^2$** | 0.016 | 0.026 | 0.017 | 0.021 | **-0.06*** | 0.063 | **-0.047*** | **-0.051*** |
| **AVA, $cm^2$** | 0.014 | 0.004 | 0.012 | 0.036 | -0.066 | 0.075 | -0.06 | -0.062 |
| **P-max, mmHg** | **4.62*** | 8.67 | **10.4*** | -0.46 | 11.3 | 5.64 | 2.92 | 3.12 |
| **P-mean, mmHg** | **3.28*** | 5.39 | **7.86*** | -1.61 | 7.5 | 4.2 | 1.93 | 1.9 |
| **VTI-ratio** | 0.017 | 0.03 | 0.037 | 0.054 | **-0.15*** | 0.041 | **-0.13*** | -0.09 |
| **Overall calcium score** | 0.015 | **0.072**** | 0.028 | 0.02 | 0.02 | -0.03 | 0.02 | 0.02 |

As demonstrated by the Table 6 several studies assessed the association between circulating levels of bone-related biomarkers and the stenosis severity in patients with AS, however with somewhat inconsistent results. Our results identified Runx2/Cbfa1 as a predictor of several measures of aortic stenosis severity (Table 5). Circulating levels of Runx2/Cbfa1 have previously been shown to serve as biomarkers for osteoblasts's anabolic function[224], and the present study for the first time associates plasma levels of this master osteogenic transcription factor with aortic stenosis.

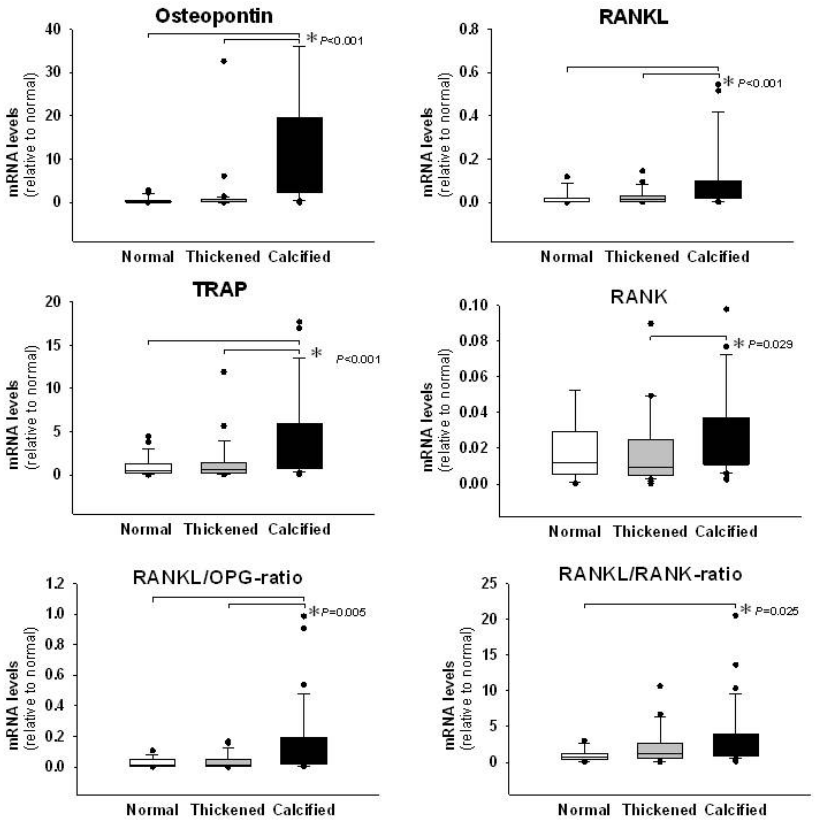



*Figure 18. Relative gene expression data (normalized for the normal area obtained from the stenotic aortic valve cusp) for OPN, RANKL, TRAP, RANK and for the ratios of RANKL/OPG and RANKL/RANK.*P<0.05 considered statistically significant.*

Table 6. Circulating bone-related biomarkers studied in patients with AS

| | **Bone-related biomarkers, human studies** | | | |
|---|---|---|---|---|
| **Biomarker measured in serum** | **Reference** | **Study population** | **Blood sample collection What was measured?** | **Main findings** |
| **MMP-9** | [225] | 22 pts with AoS | Serum (EDTA) concentrations of MMP-2, -3 and -9 pre-and postop. 2 days, 6 months and 12 months postop. | MMP-3 and -9 were elevated at two days postoperatively; MMP-3 correlated with LVEDD; MMP-2 did not change during the study period. |
| **Biomarker measured in plasma** | **Bone-related biomarkers, human studies** | | | **Main findings** |
| **BMP-2** | none | - | - | |
| **BMP-6** | none | - | - | |
| **Runx2/Cbfa1** | none | - | - | |
| **Osteocalcin (BGLAP)** | none | - | - | |
| **RANKL** | [226] | 131 adults with AoS | EDTA plasma from femoral vein and aortic root in all patients ($n$=131), and from the coronary sinus in a subgroup of 49 individuals. soluble RANKL (sRANKL) were analyzed by ELISA, which measures the free or unbound fraction of sRANKL | sRANKL was: - inversely related to age in pts with AS. sRANKL was unrelated to sex, severity of AS, presence of HF, presence of LV hypertrophy and drug therapy. A weak inverse relationship was found between the concentrations of sRANKL and OPG. |
| | [133] | 33 pts with AoS | Soluble RANKL | sRANKL concentration decreased after statin treatment in pts with AS. |
| **RANK** | none | - | - | |
| **OPG** | [226] | 131 pts with AoS | The same procedure as described at RANKL[226]. In EDTA plasma both monomeric and homodimeric forms of OPG, including OPG bound to its ligand were detected. | Circulating OPG was elevated in pts with AS, and decreased after valve repl. in pts with preop. CHF. |
| | [133] | 33 pts with AoS | Plasma OPG | OPG level before atorvastatin was statistically higher in pts with moderate valv. calcification and fell significantly after atorvastatin treatment. |
| | [227] | 136 pts with AoS | EDTA Plasma | Elevated OPG was poorly correlated with the degree of AS, but was associated with impaired cardiac function. OPG was associated with all-cause mortality in pts with symptomatic AS. |

| | | | | |
|---|---|---|---|---|
| | [228] | 38 pts with AoS+38 healthy indiv. | Plasma OPG | OPG was significantly elevated in pts with AS. |
| **γ-GT** | [154] | 64 pts with AoS | Chromatographic fraction analysis of γ-GT, f-γ-GT was the most abundant in sera compared to b-, s-, and m-γ-GT. | Total γ-GT activity in valvular tissue was negatively correlated with the extent of valve calcification;<br>both serum and tissue γ-GT levels were negatively associated with P-max. |
| **Osteopontin (SPP1)** | [129] | 23 pts with AoS, 7 controls | EDTA Plasma | Pts with modetare/severe (echocard. score of calcif.) AS hade significantly higher conc. of OPN compared with no or mild calcification of AV. |
| | [132] | 56 controls, 90 pts with ao valve sclerosis, 164 AoS pts. | Plasma | Pts with AoS and ao valve sclerosis had significantly higher OPN levels. OPN splicing variants were differentially expressed during the disease progression. |
| | [133] | 33 pts with AoS | Plasma | OPN showed a trend with decreased plasma concentrations after introducing of statin treatment; however these changes were not significant. |
| | [134] | 33 pts with AoS | EDTA Plasma | Pts with AS exhibited significantly higher conc. of OPN. NT-pro-BNP, BNP and OPN showed a direct correlation with the presence of AS. |
| | [135] | 120 pts with angina pectoris performing coronary angiogr. | EDTA Plasma | Pts with CAD had increased plasma OPN.<br>Plasma OPN levels were positively correlated with atherogenic lipid profile, hs CRP, mitral annular calcification (MAC), aortic valve sclerosis, and the number of stenosed coronary vessels in CAD pts. OPN is an independent predictor of MAC and aortic valve sclerosis and can reflect the extent of coronary stenosis. |
| | [229] | 51 pts with AS and 56 controls | EDTA Plasma OPN, the phosphorylation status of OPN was assessed. | Elevated circulating OPN levels in pts with AS; phospho-threonine levels of OPN were lower in pts with AS compared with controls. |
| **MMP-9** | none | - | - | |

Abbreviations used in Table 6: Pts: patients; LV: left ventricle; CHF: cardiac heart failure; CAD: coronary artery disease.

Although the transcriptional profile of Runx2/Cbfa1 was unaltered throughout the valve, which is in contrast with previous raports[115], however our data are in line with some previous observations suggesting that VICs may start to undergo phenotypic transdifferentiation already in the early stage of the disease (non-calcified part)[117, 230].

Plasma measures associated with osteoclastic differentiation and activity correlated with stenosis severity, such as RANKL and TRAP (Table 5), providing a first indication that osteoclast-derived biomarkers are associated with aortic stenosis.

In addition, three restrospective analysis suggested beneficial effects of nitrogen-containing bisphosphonates on AS stenosis progression inducing osteoclast apoptosis[179-181], as mentioned in the introduction section, although a recently published largest retrospective study failed to confirm those earlier reported promising results[184]. In contrast, only one study suggested that the bone resorptive activity of osteoclasts may reduce the mineral/calcium content of the valve and thus could limit the disease progression[154].

As previously demonstrated in animal models of AS, ROS are increased prior the valve dysfunction and actively mediates valvular calcification[153], firstly though induction of BMP-2 pathway[142, 202] (**paper I**), and secondly through promoting apoptosis, which together with matrix vesicles serve as a *nidus* for hydroxyapatite nucleation[80, 145]. However, it is well known from the skeletal bone physiology, that osteoblasts are redox sensitive cells and ROS might have harmful effects on them, whereas the strong oxidative milieu favours osteoclasts[219]. To confirm this notion, statins through decreasing the local inflammation could lead to decreased apoptosis and thus reduce the apoptosis induced calcification, in contrast through their anabolic effect on bone tissue[173] and as known DNA methyltransferase inhibitors trigger BMP-2 pathway and thus leading to enhanced bone formation[231]. However, the amount of active bone formation in heavily calcified aortic valves is low in comparison with the dystrophic calcification in which microfracture rate[70] is high.

The association of circulating RANKL with stenosis severity in **paper V** rather supports deleterious effects of osteclasts in AS, which is reinforced by the similar associations observed for RANKL/OPG and RANKL/RANK ratios. One possible explanation as to these findings could be proteolytic enzyme release from osteoclasts, leading to ECM degradation and valvular remodeling. Previous studies in the context of atherosclerosis indicated that RANKL leads to lesion progression with increased calcium deposition[232] which could further explain our observations of RANKL and RANK's functional consequences in AS. In agreement with this, stimulation of VICs with RANKL not only increased the proliferation the valvular structural cells[233] but also lead to significant rise in matrix calcification, nodule formation and alkaline phosphatese activity[136].

The differential spatio-temporal expression pattern of genes with osteoinductive and osteoresorptive potential suggests that the observed genes have different clinical importance during the disease initiation and progression.

It should also be acknowledged that multiple univariate associations were performed within the **paper V** and that modest testing correction through FDR (false discovery rate) resulted in somewhat lower experiment-wise significance levels compared with those obtained in individual analysis. The measured mediators should therefore not be interpreted as potential biomarkers for predicting aortic stenosis severity, but rather for their potential mechanistic involvement in the pathophysiology, which was then further explored at a tissue level. Finally, these mediators are not solely expressed in aortic valves, and it cannot be excluded that circulating levels are at least in part derived from other sources, such as skeletal bone.

In summary, **paper V** highlights a correlation between bone turnover including genes with osteinductive and osteoresorptive function and stenosis severity under *in vivo* disease development. Whereas the expression levels of genes involved in active bone formation were unaltered during the disease course, the end-stage was characterized by increased osteoclast burden with potential structural, morphological and hemodynamic consequences. In conclusion, the associations between systemic levels of Runx2/Cbfa1, RANKL, TRAP and stenosis severity highlight their critical role for stenosis development. In addition, upregulation of genes with osteoresorptive potential in stenotic valvular tissue and their correlation to stenosis severity provides new evidence for osteoclast' role in the development of AS.

# 5 CONCLUDING REMARKS

The pathomechanism of calcified aortic valve stenosis based on inflammation-dependent calcification paradigm with subsequent structural, compositional and ultimately hemodynamic consequences. The key feature of this disease in the early stages, resembling to early atherosclerotic plaque formation, is inflammation along with macrophage accumulation, endothelial- and interstitial cell activation. In advanced stages active bone formation besides dystrophic calcification containing microfractures takes place and participates in further structural changes of the valve leaflets causing stepwise increase in transvalvular pressure gradient as the stenosis progresses.

Aortic valve stenosis is an important health problem and has become the most common indication for surgical valve replacement. To date there is no medical treatment other than surgical valve replacement for this disease, and therefore it is crucial to find a potential treatment for this progressive disorder.

The proof-of-concept of this thesis was based on the assumption that the leukotriene pathway with potent proinflammatory properties might be involved in the pathogenesis of aortic valve stenosis with a potentially direct association coupled to disease progression. We have established a unique macroscopic dissection technique, which allowed modeling the *in vivo* disease development, representing the entire disease spectrum from early signs to more pronounced morphological changes.

This thesis presents a new approach of the molecular understanding of stenosis development integrating findings of molecular research using quantitative gene expression data with clinical variables in terms of echocardiographic parameters. In addition, to further characterize the pathobiological process, the thesis also includes results of mechanistic experiments using *in vitro* cell culture model on isolated valvular interstitial cells and vascular smooth muscle cells.

The summary of the findings presented in this thesis is depicted on Figure 19.

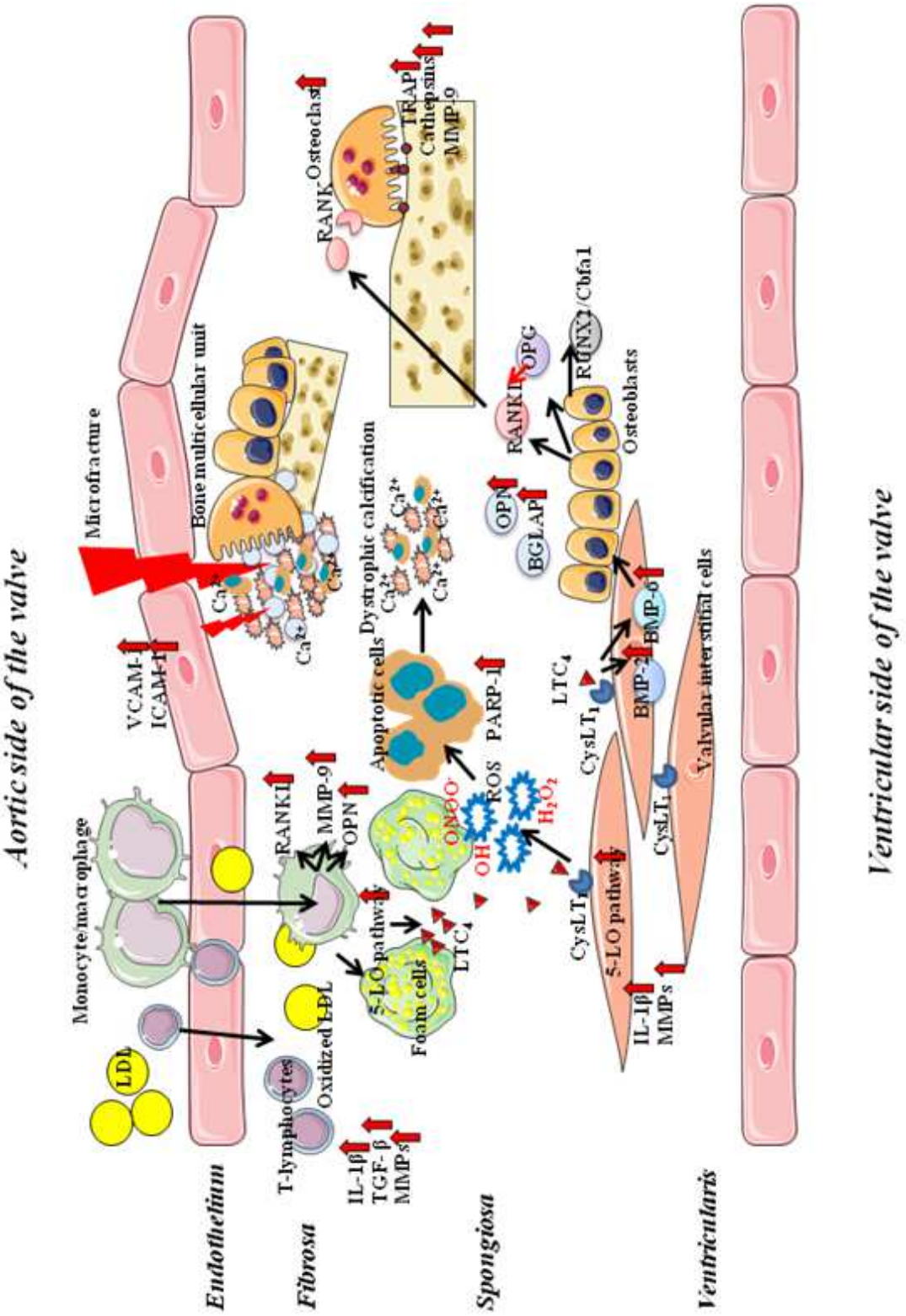
The inflammatory environment within the affected aortic valve along with subendothelial accumulation of oxidized LDL stimulates the 5-lipoxygenase pathway in macrophages leading to production of potent inflammatory mediators, leukotrienes. In addition, to further enhance the local inflammatory response, valvular interstitial cells, representing a highly plastic cell population, through epigenetic modification will be able to produce 5-lipoxygenase, and thus contribute to amplification of the inflammation. The locally produced $LTC_4$ through activation of $CysLT_1$ receptor, localized on immune competent-and interstitial cells leads to a cascade of events promoting ROS production, dissipation of mitochondrial membrane potential, increase in $CysLT_1$ receptor expression and sensitivity with subsequent altered gene expression profile towards atherogenic properties. All those events coupled to increase in nuclear calcium signal in response to $LTC_4$ stimulation. In addition, $LTC_4$ is a potent inducer of the osteogenic morphogenes BMP-2 and BMP-6, linking inflammation and active cuspal calcification together. Furthermore, the locally produced ROS triggers the nuclear enzyme PARP-1, which along with decreased mitochondrial membrane potential and overt formation of intracytoplasmatic vacuoles facilitates apoptotic cell death. The apoptotic bodies and matrix vesicles serve as a *nidus* for hydroxyapatite nucleation thus taking part in dystrophic calcification. The spatio-temporal distribution of genes with osteoinductive and osteoresorptive potential is different at different stages of the disease according to our observations. Whereas osteoblast-associated genes seem to be unaltered throughout the natural course of the stenosis development, the strong oxidative milieu favors the presence of osteoclasts. The active influence of osteoclasts, possibly through their potent proteolytic enzyme release might rather promote further calcification in diseased valve.

These above findings were confirmed at systemic level. Plasma measures associated with osteoclastic differentiation and activity correlated with stenosis severity, such as RANKL and TRAP, providing a first indication that osteoclast-derived biomarkers are associated with aortic stenosis. In addition, the active influence of Runx2/Cbfa1, a master transcription factor for terminal osteoblastic differentiation, to stenosis severity was consequent in all our analysis. This confirms the associations between systemic levels of Runx2/Cbfa1, RANKL, TRAP and stenosis severity and highlights their critical role for stenosis development. In

addition, upregulation of genes with osteoresorptive potential in stenotic valvular tissue and their correlation to stenosis severity provides new evidence for their role in the development of aortic stenosis.

In summary, this new concept characterizing the entire disease spectrum from early phases to end-stage might reveal new therapeutic approaches. Translational implication of our data suggests possibility for pharmacological intervention using leukotriene receptor antagonists with a potential to retard the hemodynamic progression.

*Figure 19. Overview of the pathomechanism of AS. Concluding remarks, explanation is in the text.*



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