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Gene expression differences in cardiovascular disease

Genexpressionsunterschiede in kardiovaskulären Erkrankungen

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1. Introduction

In 2008 cardiovascular disease accounted for 30% of global mortality (17.3 million deaths), thereby representing the worldwide leading cause of death[1]. This thesis contains three studies about gene expression differences in cardiovascular disease. The cardiovascular diseases in question are dissections of the ascending aorta, ascending aortic aneurysms associated with bicuspid aortic valve as well as atrial fibrillation. The aim of each study is to contribute to the understanding of the pathogenesis of the particular disease and give future tools in diagnostics.

1.1 Ascending aortic aneurysms and dissections

An aortic aneurysm is a permanent localized dilatation of the aorta with an at least 50% increase in diameter[2]. In a population based cohort study the overall incidence of thoracic aortic aneurysm was 10.4 per 100,000 person-years[3]. Thoracic aneurysms can occur in the ascending aorta, the aortic arch, the descending aorta or extend to the abdominal aorta as thoracabdominal aneurysm. With a frequency of 60% the ascending aorta is most often affected[4]. While aortic aneurysms are generally a benign condition, they can, with growing diameter, give rise to catastrophic events like acute aortic dissection or aortic rupture. At an average diameter of 6 cm the probability for dissection or rupture of the ascending aorta rises from 6.6% to 36.2%, thus marking a point where surgical intervention seems necessary[5].

An aortic dissection occurs when a tear in the inner aortic layer (the *intima*) induces a splitting of the subsequent layer (the *media*) and the creation of a false lumen by the blood stream. From here the dissection can propagate in anterograde or retrograde direction[6, 7]. A further re-entrance tear may allow the blood to circulate through the false lumen leading to the formation of a double channel aorta with a high probability of aortic rupture[8, 9].

Thoracic aortic dissections are classified according to the site of the initial tear. After Stanford, dissections involving the ascending aorta are classified as type A dissection, whereas dissections not involving the ascending aorta are referred to as type B dissection[10]. Among 464 subjects with acute aortic dissection the mean age

was 63 years, with a male dominance of 65.3% and a 62.3 percentage of type A dissections[11].

Thoracic aortic dissection is a severe cardiovascular event with the urgent need for treatment. The overall in-hospital mortality for acute aortic dissection is 27.4%, with a mortality of 32.5% for type A dissections[11, 12]. The mortality 6 hours after admission is 22.7 %, rising to 33.3%, 50% and 68.2% after 12, 24 and 48 hours respectively[13].

The molecular mechanisms involved in the pathogenesis of thoracic aortic dissection are multifarious and generally any condition that weakens the aortic wall can contribute to the event. One of these conditions is cystic medial necrosis, a histologic abnormality commonly found in aneurysms and dissections of the thoracic aorta and characterised by the fragmentation of the elastic lamina, the loss of vascular smooth muscle cells and the accumulation of basophilic ground substance[14].

Aneurysms and dissections of the thoracic aorta are highly associated with hereditary influence, and genetic syndromes like Marfan syndrome, Turner syndrome, Ehlers-Danlos syndrome or Loeys-Dietz syndrome are major predisposing factors[9, 15, 16]. However only 5% of aortic dissection cases are associated with Marfan syndrome and even less with other syndromes, which means the vast majority of cases is non-syndromic[17]. In a study by Coady and colleagues 19.3% of 135 individuals with non-syndromic thoracic aortic aneurysms and dissections displayed a familial pattern of inheritance, while the rest was sporadic. Of the familial cases, 38.5% showed an autosomal dominant mode of inheritance, 23.1% displayed an autosomal dominant or X-linked mode of inheritance and 26.9% were in agreement with a recessive mode of inheritance[18]. Many forms of thoracic aortic aneurysms and dissections share a perturbation of the transforming growth factor β (TGF-β) signalling pathway, known to play an important role in cellular proliferation, differentiation and extracellular matrix production[16]. In Marfan syndrome the *Fibrillin 1* gene is mutated, coding for an extracellular matrix component which participates in the regulation of TGF-β bioavailability[19-21]. Other members of this cytokine signalling pathway are mutated in Loeys-Dietz syndrome (*TGF-β receptor 1* or *2*) and a syndrome caused by mutations in the *Smad 3* gene, coding for a downstream target of TGF-β[15, 22]. Proteins of the extracellular matrix or involved in extracellular matrix assembly play a part in the pathogenesis of thoracic aortic aneurysms and dissections, probably regulated by the TGF-β pathway. Matrix metalloproteinases (MMPs) and tissue inhibitors of

metalloproteinase (TIMPs) facilitate remodelling of the extracellular matrix and are dysregulated in thoracic aortic aneurysms and dissections[23]. Mutations in the *fibulin 4* gene result in a phenotype that comprises thoracic aortic aneurysm in human and mice[24-26]. Fibulin 1 and Decorin are other extracellular components that are dysregulated in aortic dissection without Marfan syndrome[27]. Disturbances in the contractile apparatus also promote the formation of thoracic aortic aneurysms and dissections. Particularly mutations the gene coding for smooth muscle α-actin (*ACTA2*) are made responsible for 14% of inherited ascending thoracic aortic aneurysms and dissections[28]. The *myosin heavy chain 11* gene (*MYH11*) on the other hand, is mutated in families which display a phenotype of thoracic aortic aneurysms and dissections paired with a patent ductus ateriosus[29, 30].

As a part of this study the expression of the *high mobility group AT-hook 2* (*HMGA2*) gene was to be analysed in thoracic aortic dissection tissue and compared to control tissue. The *HMGA2* gene codes for a DNA binding transcription factor mainly expressed during embryogenesis but reactivated in numerous human tumors[31, 32]. In cancer biology the HMGA2 protein acts as a downstream target to TGF-β signalling and elicits the transition of epithelial cells to motile mesenchymal cells, a process referred to as *epithelial to mesenchymal transition* (EMT)[33]. It was therefore hypothesised that the abnormal TGF-β signalling in thoracic aortic dissections could lead to an increase in *HMGA2* expression.

1.2 The bicuspid aortic valve

The bicuspid aortic valve (BAV) is a common congenital cardiac anomaly. It is the most frequent malformation of the heart or great vessels with a prevalence of 0.9% to 2% in the general population and a strong male predilection[34]. In contrast to the normal tricuspid aortic valve (TAV) it is comprised of two cusps, often of unequal size. The larger cusp frequently features a raphe, a fibrous ridge that represents the site of congenital fusion of two cusps[35]. The cusps are attached to the aortic sinuses, which are termed after the position of the coronary arteries as left-coronary, right-coronary or non-coronary sinus. Accordingly BAV are classified by the spatial position of the fused cusps as L-R (fusion between left- and right-coronary sinus),

R-N (fusion between right- and non-coronary sinus) and N-L (fusion between non- and left-coronary sinus) (Figure 1)[36, 37].

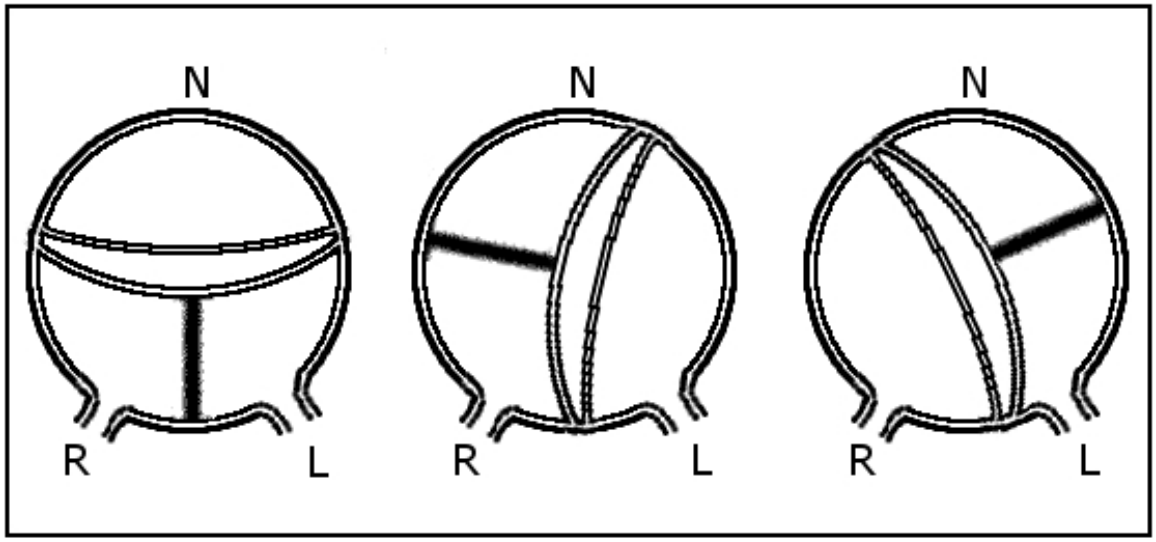

Figure 1: Different types of BAV. Fusion between the left- and right-coronary sinus (L-R BAV) is shown on the left, fusion between the right- and non-coronary sinus (R-N BAV) is shown in the middle and fusion between non- and left-coronary sinus (N-L BAV) is pictured on the right. The raphe is presented as black line. L: Left-coronary sinus; N: Non-coronary sinus; R: Right-coronary sinus (Figure based on figure 3 from reference 38).

These BAV types are not equally distributed. The L-R type is most common with a frequency of 74% to 86%, followed by the R-N type with a frequency of 12% to 24% and the L-N type which contributes 0.5% to 3% of BAV cases[36-39]. Fernández et al. demonstrated a different developmental origin of the L-R and R-N type BAV in a study that compared inbred syrian hamsters and transgenic mice[40].

The BAV gives often rise to an underlying aortic valve disease, such as aortic valve stenosis or aortic valve insufficiency. In a necropsy series of 85 BAV cases, 61 (72%) suffered from aortic valve stenosis, often accompanied by aortic valvular calcification. Eleven of the 85 cases (13%) had aortic valve insufficiency without stenosis and only 13 of 85 (15%) had a normal functioning BAV without any further disease[34]. Two larger clinical studies stated a frequency of 51% to 75% for aortic valve stenosis, 13% to 38% for aortic insufficiency, 9% to 10% for combined stenosis and insufficiency and 1% for a normal BAV[36, 38]. Since both of these studies only contained cases of aortic valve replacement, the frequency of a normal BAV is probably underestimated due to the lesser need for surgery in these patients. Other

diseases commonly associated with the BAV are infective endocarditis as well as aneurysms and dissections of the aortic root and ascending aorta[35, 41-44]. Dilatations and aneurysms of the ascending aorta occur frequently in patients with BAV. Nistri and colleagues found an enlargement of the aortic root in 52% of young men with a normal BAV[43]. Likewise aortic dissections, as a consequence of aortic aneurysms, are found more commonly in individuals with BAV. Several studies found an increase in BAV prevalence among patients with aortic dissection, ranging from 7.5% to 14% for dissections of the ascending aorta[41, 42, 45]. Compared to the BAV prevalence in the general population these studies estimated a five- to nine-fold higher occurrence of BAV in cases with aortic dissection[34, 45], or the other way round, the presence of a BAV increases the risk for aortic dissection by the factor nine[41]. Additionally aortic dissection occurred at a younger age in BAV than in TAV patients (54 vs 62 years)[42]. Thus the existence of a BAV can be seen as a severe risk factor for acute aortic dissection. Moreover the commonness of BAV combined with the mortality caused by accompanying diseases like aortic valve stenosis, aortic valve insufficiency or aortic dissection have provoked Ward to the statement: “This condition may be responsible for more deaths and morbidity than the combined effects of all other congenital heart defects”[46].

Despite intensive research on the field, the genetic basis of the BAV is still poorly understood. In a work by Huntington et al. 9.1 % of BAV first degree relatives had a BAV themselves, with a distribution within the families that is compatible with an autosomal dominant pattern of inheritance with reduced penetrance[47]. A further study of 309 probands with BAV and their relatives detected a BAV prevalence of 24% in this group and accordingly estimated a heritability of 89%[48]. Up to date it has not been possible to explain the wide spectrum of BAV manifestations with a single gene defect, which indicates that the BAV is most likely a complex and heterogeneous disease. Mutations in the signalling and transcriptional regulator *NOTCH1* caused BAV and severe aortic valve calcification in two families but can also be observed in approximately 4% of sporadic cases[49, 50]. Furthermore the expression of the *UFD1L* gene and the according protein, playing a role in the cardiac outflow tract during embryogenesis, are down-regulated in BAV tissue[51]. Family based linkage analysis has revealed three loci on chromosomes 18q, 5q and 13q responsible for BAV, but no gene was identified[52], while a computational approach identified SNPs within the

AXIN1-PDIA2 locus, the *endoglin* gene and on chromosome 3 using a genome wide SNP data set[53].

Data from animal models bestow additional insight into the pathogenesis of BAV. Mice deficient of the nitric oxide generating enzyme eNOS demonstrate a high frequency of R-N type BAV[40, 54]. Deletion of the zinc finger protein GATA5 results in a 25% incidence of R-N type BAV in transgenic mice[55] and mice with heterozygous mutations in the cardiac homeobox gene *Nkx2-5* or mice deficient of the homeobox gene *Hoxa1* develop a BAV as part of several severe cardiac malformations[56, 57].

Irrespective of the pathogenesis is the question why the BAV is accompanied by aortic aneurysm with such a high frequency. A connection between the presence of a BAV and aneurysm formation has been suspected for long and today two main theories are wildly discussed[58, 59]. The first theory focuses on the assumption that the genetic defect responsible for the BAV also weakens the aortic wall and therefore facilitates the occurrence of aortic aneurysm. There is evidence in the fact that dilatated BAV aortas frequently feature the histologic abnormality termed cystic medial necrosis, which is also found in aortic aneurysm not linked to BAV[60]. The second theory proposes a weakening of the aortic wall by laminar shear stress caused by the diseased BAV. This theory is supported by the asymmetrical distribution of extracellular matrix protein expression and smooth muscle cell apoptosis within the proximal BAV aorta, indicating shear stress induced changes at certain sites[61, 62].

One aim of this study was to analyse the expression of the eNOS protein in different areas of proximal BAV aortas with aortic aneurysm. While eNOS is also linked to the pathogenesis of BAV in mice, Aicher et al found a decreased expression in aortic tissue of BAV aneurysm[63]. They suggested that eNOS plays a role in BAV aneurysm formation, since expression and activity of the enzyme can be regulated by fluid shear stress[64, 65]. We therefore wanted to verify if the eNOS protein is asymmetric expressed at different aortic locations compared to TAV controls. This would indicate that the presence of a BAV could alter the laminar shear stress in the proximal aorta and influence gene expression.

1.3 Atrial fibrillation

Atrial fibrillation is a form of cardiac arrhythmia where the atrial activity escapes the control of the sinoatrial node and is replaced by disorganized rapid excitations[66]. The overall prevalence of atrial fibrillation is 0.4% to 0.95% in the general population, 5.5% in patients over 55 years with up to 9% or 17.8% at over 80 years or over 85 years respectively, thus rendering it the most common form of arrhythmia[67-69]. Additionally the prevalence is estimated to double by the year 2050, due to the growing population in elderly people[68]. The fast excitations of the atrium provoke a loss of atrial contraction which leads to stasis of the blood in the atria and thereby increases the risk of thromboembolic events[66]. Hence 15% to 25% of stroke can be attributed to the arrhythmia with stroke in atrial fibrillation being more severe than other forms[70, 71]. Overall stroke and other cardiovascular events lead to a doubling of mortality in atrial fibrillation[71, 72].

Atrial fibrillation is typically triggered by ectopic foci of anomalous electric activity, which can originate from the pulmonary veins or from abnormal activity of the automatic nervous system[73-76]. The ongoing arrhythmia can induce electrical remodelling of the atria through alterations in ion channel functions and structural remodelling via increased fibrosis. These mechanisms can create re-entrant circuits of conduction either by shortening of the action potential duration and refractory period or by causing conduction delay through fibrotic areas[75, 77]. Normally an ectopic action potential can not activate neighbouring tissue zones which are still in the refractory period. A re-entrant circuit arises when fibrotic areas of conduction delay cause the impulse to propagate through an alternative, longer pathway and hit the neighbouring zone just after the refractory period ends, thereby causing a circuit of rapid activation. Multiple re-entrant circuits can exist in the atria, causing an irregular excitation[66, 77, 78].

Electrical remodelling of the atria during atrial fibrillation can facilitate to sustain the arrhythmia. The cellular Ca^{2+} metabolism plays an important part in this process, since the rapid excitations of the cells lead to an increased and potentially cytotoxic Ca^{2+} uptake[79]. As a consequence, the activity and expression of the inward L-type Ca^{2+} current ion channel (I_{ca}) is reduced, leading to a decrease in action potential duration and refractory period and thereby promoting the appearance of further re-entrant circuits[80-82].

The fact that atrial fibrillation can sustain itself by electrical and structural remodelling offers a classification system based on the duration of the arrhythmia. In the 2010 atrial fibrillation guidelines of the European Society for Cardiology (ESC) episodes of 48 hours or less are described as paroxysmal atrial fibrillation, episodes that last longer than 7 days or require medical treatment to be terminated are classified as persistent atrial fibrillation and arrhythmias that lasted for over one year are considered to be long-standing persistent. An arrhythmia is viewed as permanent when it has been accepted by the patient and heart rhythm control is not utilized any more[83].

Atrial fibrillation is an at least in part heritable disease, since parental atrial fibrillation vastly increases the risk of affected offspring[84]. Up to date three loci, which are associated with the disease, have been identified by genome wide association studies. These are located upstream of the transcription factor gene *PITX2*, and within the transcription factor gene *ZFHX3* and the potassium channel gene *KCNN3*[85-88]. Various other mutations, mainly in genes coding for potassium or sodium channels, have been linked to familial forms of atrial fibrillation, but the mutations in the genes coding for the gap junction protein connexin 40, the signalling molecule atrial natriuretic peptide and a down-regulation of the extracellular matrix component fibulin-1 are also related to the disease[89-92].

In recent years the importance of micro RNA (miRNA) mediated post-transcriptional regulation in the pathogenesis of atrial fibrillation has become aware[93]. Several miRNAs have been associated with atrial fibrillation, including miRNA 1, which slows conduction and depolarizes the cell membrane by targeting the potassium channel gene *KCNJ2* and the gene *GJA1*, coding for connexin 43[94]. The miRNA 328 has been shown to perturb the cellular Ca^{2+} metabolism by targeting the calcium channel genes *CACNA1C* and *CACNB1*[95].

A further miRNA linked to arrhythmias in mice is miRNA 208a[96]. Since this miRNA has not yet been analysed in human atrial fibrillation, it was aimed to quantify the miRNA 208a expression in left atrial appendage tissue of human atrial fibrillation patients as a part of this study. Samples of paroxysmal, persistent and long-standing persistent atrial fibrillation tissue were planed to be used for this study to investigate the expression changes during the time course of the ongoing arrhythmia.

2. Materials and methods

2.1 Protein extraction from tissue samples

For the extraction of the cytoplasmatic protein fraction from snap frozen tissue samples RIPA-buffer (containing 150mM NaCl, 50mM Tris HCl, 0.5% deoxycholic acid and 1% NP-40) was used as cell lysis buffer. First tissue samples of approximately 100mg were homogenised in 0.5ml RIPA-buffer containing protease inhibitor solution (Roche, Mannheim, Germany). The lysate was incubated on ice for 45min and subsequently centrifuged for 10min at 12000xg and 4°C. The supernatant containing the proteins was stored at -20°C.

2.2 Measuring of whole protein concentration

The whole protein concentration was determined using the BCA protein assay (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. The micro-plate procedure was utilised and all calculations were done in Excel.

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The proteins were separated on NuPAGE 10% Bis-Tris gels using the Xcell SureLock Mini-cell (both Invitrogen, Darmstadt, Germany). For publication I between 1µg and 50µg of whole protein lysate were used as starting amount, whereas for publication II 20µg of protein lysate were used as starting amount.

2.4 Western blot

The proteins were transferred to a nitrocellulose membrane with the Blot module of the Xcell SureLock Mini-cell (Invitrogen, Darmstadt, Germany) for 1h at 30V. Afterwards the membrane was incubated with blocking buffer (5% BSA in TBS-T) at 4°C over night, washed thrice in TBS-T and then incubated with the primary antibody diluted in blocking buffer for 1h. After an additional wash step the membrane was treated with alkaline phosphatase conjugated secondary antibody in blocking buffer and then washed again and incubated with NBT/BCIP solution (Roche, Mannheim, Germany) until bands developed. The intensity of the bands was quantified with the imagej software[97].

For publication I primary antibodies against HMGA2 (sc-30223) and SNAI1 (sc-28199) (both by Santa Cruz Biotec, Heidelberg, Germany) were used at a dilution of 1:200, whereas the secondary antibody bovine anti-rabbit IgG-AP (sc2372, Santa Cruz Biotec, Heidelberg, Germany) was applied in a dilution of 1:5000. In publication II primary antibodies against eNOS (sc-654), GAPDH (sc-47724) (both by Santa Cruz Biotec, Heidelberg, Germany) were utilised at a dilution of 1:200 while ACTB (NB600-501, Novus Biologicals, Herford, Germany) was used at a dilution of 1:15000. The secondary antibodies used in this publication were bovine anti rabbit IgG-AP (same as in publication I) and Goat Anti-Mouse-AP (G21060, Invitrogen, Darmstadt, Germany) in a dilution of 1:7500.

2.5 Cell culture

The vascular smooth muscle cells (VSMCs) used in publication II originated from the human aortic *tunica media*. The cells were immortalised after transfection with pSV40-dN-plasmid DNA. They were grown in cell culture medium (TC199 with Earle's salts supplemented with 20% fetal bovine serum, 200 U/mL penicillin, 200 *µ*g/mL streptomycin) at 37°C and 5% CO_2 until approximately 80% confluency and where then passaged using TrypLE (Invitrogen, Darmstadt, Germany) to detach the cells.

Additionally primary human aortic endothelial cells (HAECs) (Lonza, Cologne, Germany) were grown in enthodelial cell growth medium-2 (Lonza, Cologne, Germany) and kept at 37°C and 5% CO_2. These cells were also passaged at 80% confluency using TrypLE.

2.6 Inhibition of nitric oxide synthase activity

The activity of nitric oxide synthases was inhibited with N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma-Aldrich, Seelze, Germany). The L-arginine analogue L-NAME can act as an unspecific inhibitor of the three nitric oxide synthase isoforms (nNOS, eNOS and iNOS)[98, 99]. Therefore VSMCs cells and HAECs were grown to 90% confluency and afterwards incubated in serum-free medium for 24h. The cells were then treated with 1mM L-NAME for 1h after which the cellular proteins were extracted.

2.7 Protein extraction from cells

The cells were washed once with ice-cold PBS before 500µl of RIPA-buffer/protease-inhibitor solution were added to a 25cm^2 cell culture flask and the cells were detached using a cell scraper. The cell solution was afterwards incubated on a shaker on ice for 35min and subsequently centrifuged at 14200xg and 4°C for 20min. The supernatant containing the cytosolic protein fraction was collected and stored at -20°C.

2.8 Proteome profiler array

The quantity of 35 apoptosis related proteins was measured with the Proteome Profiler Array for human apoptosis (R&D Systems, Wiesbaden-Nordenstadt, Germany). For this purpose 200µg of L-NAME treated cytosolic proteins were analysed according to the manufacturer's instructions. However the protocol was changed as an antibiotin alkaline phosphatase conjugated secondary antibody (Sigma-aldrich, Munich, Germany) was used for the detection in a 1:10000 dilution together with NBT/BCIP Solution (Roche, Mannheim, Germany). The intensity of the single protein spots was quantified with the imagej software and normalised to the positive control.

2.9 RNA isolation

RNA isolation from tissue samples was carried out with the TRIzol reagent (Life Technologies, Darmstadt, Germany) according to the protocol. For tissue homogenisation approximately 100mg of snap frozen tissue were lysed with 1ml of TRIzol reagent in a tissue lyser (Qiagen, Hilden, Germany). Concentration and purity of the isolated RNA were determined with a BioPhotometer (Eppendorf, Hamburg, Germany).

2.10 cDNA synthesis

For synthesis of cDNA from microRNA the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany) was used according to the manufacturer's instructions. The reverse transcription primers for microRNA 208a were from assay ID 000511 and the primers for RNU48 were from assay ID 001006 (both Applied Biosystems, Weiterstadt, Germany).

2.11 Real-time PCR

The relative quantification of the MicroRNA 208a expression was carried out on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Weiterstadt, Germany). The snRNA RNU48 served as endogenous control, assay IDs for MicroRNA 208a and RNU48 are the same as in cDNA synthesis and universal PCR mater mix (Applied Biosystems, Weiterstadt, Germany) was used. All samples were run in triplicates with controls without reverse transcriptase for every sample and no template controls for every PCR reaction. The PCR profile contained an initial step of 2min at 50°C, followed by a 10min denaturation step at 95°C, and 40 cycles of 15sec denaturation at 95°C and 1min annealing/elongation at 60°C. The relative amount of the microRNA 208a expression was calculated using the $\Delta\Delta$CT method[100].

2.12 Statistical analysis

The statistical analysis was done in Microsoft Excel (Microsoft, Redmond, USA) with the winSTAT add-in (R.Fitch Software, Bad Krozingen, Germany) and SPSS (IBM, Armonk, USA). Data are usually presented as mean ± standard deviation or as relative percentage. Differences between independent variables were tested with the two-sided Mann-Whitney U-test in all three publications, whereas the Wilcoxon signed-rank test was used to analyse dependent variables in publication II. Repeated measurement two-way ANOVA was used to analyse the differences in the proteome profiler arrays (publication II), which consisted of two replicates. The two-sided Fisher's exact-test was used to compare categorical variables and the association between different parameters was analysed by Pearson's correlation coefficient. Significant differences were assumed at $p < 0.05$.

3. Results

3.1 Publication I: Upregulation of the high mobility group AT-hook 2 gene in acute aortic dissection is potentially associated with endothelial-mesenchymal transition

The *high mobility group AT-hook 2* (*HMGA2*) gene encodes for a non-histonic DNA-binding protein. Members of the HMGA protein family can bind to AT-rich sequences in the minor groove of DNA, cause conformational changes of the DNA and thereby regulate transcription of other genes[101-103]. They are normally expressed during embryogenesis but not in differentiated adult cells, with reexpression occurring in various tumors[31, 32, 104].

Activation of the TGF-β pathway can induce *HMGA2* expression via Smad-signalling. The HMGA2 protein then proceeds to regulate the expression of *SNAI1* and *TWIST*, two genes required for epithelial to mesenchymal transition (EMT)[33, 105, 106]. Throughout this process, which is critical to embryogenesis but also contributes to tumor invasiveness and metastasis, epithelial cells are converted to mesenchymal cells[107]. In fact the ability of HMGA2 to induce EMT has been demonstrated in various cancers[108, 109].

Interestingly many forms of ascending aortic aneurysms and dissections have a dysregulation of the TGF-β pathway in common, leading to an increase in TGF-β signalling in most cases[16]. It is therefore possible that this would provoke a *HMGA2* reexpression in patients suffering from ascending aortic aneurysms or dissections, which could in turn elicit a form of EMT. Accordingly the *HMGA2* expression in 26 cases of ascending aortic aneurysms and 19 cases of type A acute aortic dissection was quantified in this study. Furthermore the *in situ* protein expression of HMGA2 and the EMT-markers SNAI1 and Vimentin was analysed by immunohistochemistry and the expression of the *let-7d* microRNA, which targets *HMGA2*, was also quantified.

The *HMGA2* expression in ascending aortic aneurysm was modest (8.6±9.7-fold expression of the calibrator), whereas it was high in acute aortic dissection (193.1±272.8). The difference between acute aortic dissection and samples without

dissection (ascending aortic aneurysms with samples from Marfan syndrome and control samples from aortic valve tissue) was highly significant ($p = 1.4*10^{-7}$). Increased protein amounts of HMGA2, SNAI1 and Vimentin were detected mainly in the endothelial cells of the *vasa vasorum*, although with seven of eleven cases being positive in acute aortic dissection for HMGA2 and SNAI1 and ten of nineteen cases being positive for Vimentin, compared to two of eleven cases and two of ten cases, respectively, the difference between acute aortic dissection and non-dissecting tissue was not significant ($p = 0.08$ for HMGA2 and SNAI1, and $p = 0.1$ for Vimentin). The correlation between the *let-7d* expression and the *HMGA2* expression in the same patients was poor, indicating that this microRNA does not regulate *HMGA2* in acute aortic dissection.

Taken together these results suggest an increased *HMGA2* expression in acute aortic dissection patients but not in ascending aortic aneurysms or Marfan syndrome. This *HMGA2* overexpression seems to trigger EMT in the endothelial cells as indicated by the presence of SNAI1 and Vimentin.

Upregulation of the *high mobility group AT-hook 2* gene in acute aortic dissection is potentially associated with endothelial-mesenchymal transition

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- Part of the study design
- Validation of the used antibodies in western blot
- Statistical analysis
- Co-writing of the manuscript

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Histology and Histopathology

Cellular and Molecular Biology

Upregulation of the *high mobility group AT-hook 2* gene in acute aortic dissection is potentially associated with endothelial-mesenchymal transition

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Summary. *The high mobility group AT-hook 2 (HMGA2)* gene is proposed to regulate the genes involved in the epithelial-mesenchymal transition (EMT). One form of EMT is endothelial-mesenchymal transition (EndMT). We analyzed the expression profile of the *HMGA2* gene in different human aortic diseases.

Aortic specimens were collected from 51 patients, including 19 with acute aortic dissection, 26 with aortic aneurysm, two with Marfan syndrome and four aortic valves. Quantitative real-time polymerase chain reaction was carried out for *HMGA2* and immunohistochemical analyses were performed for *HMGA2*, SNAI1, Vimentin, CD34, MKI-67 and TGFB1. The expression of *let-7d* microRNA, which is assumed to play a role in the regulation of *HMGA2*, was also quantified.

The level of *HMGA2* gene expression was significantly higher in acute aortic dissection compared with all the other samples (193.1 vs. 8.1 fold normalized to calibrator, $P<0.001$). The immunohistochemical investigation showed that HMGA2, SNAI1, and Vimentin proteins were mainly detected in the endothelial cells of the *vasa vasorum*.

The *HMGA2* gene is upregulated in acute aortic dissection. This is the first report describing a link between HMGA2 and acute aortic dissection. The HMGA2, SNAI1 and Vimentin proteins were mainly detected in the endothelium of the *vasa vasorum*. It seems that *HMGA2* overexpression in acute aortic dissection occurs in a let-7d-independent manner and is associated with EndMT of the *vasa vasorum*.

Key words: HMGA2, Acute aortic dissection, EMT/EndMT, Vasa vasorum

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Introduction

The *high mobility group AT-hook 2* (*HMGA2*) gene has the ability to bind to AT-rich DNA sequences and to induce global changes in chromatin structure and thereby regulate transcription (Aravind and Landsman, 1998; Merika and Thanos, 2001). It mediates epithelial-mesenchymal transition (EMT) in response to transforming growth factor beta (TGFB)-induced Smad pathway (Valcourt et al., 2005; Thuault et al., 2006; Zeisberg et al., 2007). Epithelial-mesenchymal transition describes a process that reorganizes epithelial cells into migratory mesenchymal cells (Valcourt et al., 2005). This process is critical to normal embryogenesis and is a defining structural feature of organ development. One form of EMT is endothelial-mesenchymal transition (EndMT), which occurs during the embryonic development of the heart (Zeisberg et al., 2007). Among the modulators of EMT is snail homolog 1 (SNAI1), a transcriptional repressor, which is regulated by *HMGA2* (Thuault et al., 2006). The SNAI1 protein acts as a strong repressor of epithelial-specific genes such as *E-cadherin,* thereby promoting EMT, and it is considered to be a marker of EMT (Peinado et al., 2004; Lee et al.,

2006). During aortic and heart valve maturation SNAI1 is required for TGFB-induced EndMT of embryonic stem cells (Kokudo et al., 2008).

Normally, the level of *HMGA2* expression is very high during embryonic development, whereas it is low, or even undetectable, in differentiated adult cells (Chiappetta et al., 1996; Rogalla et al., 1996). The expression of *HMGA2* is controlled by the microRNAs of the let-7 family, which target its 3'UTR (Lee and Dutta, 2007). Reactivation of *HMGA2* due to cytogenetically detectable aberrations of chromosomal region 12q13-15 is found in a variety of tumors of mesenchymal origin (Abe et al., 2003; Borrmann et al., 2003; Fusco and Fedele, 2007; Belge et al., 2008). Pathogenetically, re-expression of *HMGA2* has also been implicated in the formation of arteriosclerotic plaques and restenosis (Zhou et al., 1995; Chin et al., 1999; Anand and Chada, 2000). However, the involvement of *HMGA2* in acute aortic dissection (AAD) is unknown. Acute aortic dissection is a life-threatening disease with high morbidity and mortality rates, and is generally an unpredictable event (Wheat, 1987; Abbara et al., 2007; Isselbacher, 2007). People commonly at risk of this disease include those with connective tissue disorders such as Marfan syndrome, Ehlers-Danlos syndrome and Erdheim Gsell medial necrosis, but also bicuspid aortic valve. Bicuspid aortic valve is the most common form of congenital heart disease with a frequency of concomitant aortic dilatation of 45-50% (Dietz et al., 1994; Silverman et al., 1995; Beroukhim et al., 2006). Indeed, patients with bicuspid aortic valve are at an increased risk of developing ascending aorta complications and AAD (Januzzi et al., 2004). The pathophysiological mechanism in AAD is most likely multifactorial (Guo et al., 2007; Mohamed et al., 2008).

Since HMGA2 is known as a crucial factor in cardiogenesis, a dysregulation may cause alterations in adult vascular tissues (Monzen et al., 2008). Therefore, we examined *HMGA2* expression in different aortic diseases. Acute aortic dissection patients, who do not suffer from Marfan syndrome, were compared with patients suffering from thoracic aortic aneurysm or Marfan syndrome. We studied the *let-7* miRNA expression in patients with AAD and compared it to the expression of *HMGA2* to reveal a possible dysregulation of *HMGA2* and its inhibitory miRNA. Furthermore the protein levels of *HMGA2*, SNAI1, Vimentin and TGFB were investigated by immunohistochemistry.

Material and methods

Tissue samples

The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all patients. In cases of aortic valve or ascending aorta replacement, diseased aortic tissue was collected during surgery and carefully divided into two parts. One part was immediately snap-frozen in liquid nitrogen and preserved at -80°C to be used later for gene expression and protein analyses. The second part was fixed in 4.5% paraformaldehyde and then embedded in paraffin. The patients' demographics are shown in Table 1.

RNA isolation, reverse transcription, and qRT-PCR for HMGA2 measurement

Total RNA was purified according to the RNeasy® Mini Kit protocol for isolation of total RNA from heart, muscle and skin tissue, including on-column DNaseI digestion and homogenization with TissueLyser (Qiagen, Hilden, Germany). The input amount of tissue was about 5-10 mg. The RNA was quantified and 5 μg RNA was treated with a second DNaseI (6.75 U) digestion for 15 min at room temperature and a cleanup, according to the RNeasy Mini Kit protocol, to remove as much contaminating DNA as possible. Up to 250 ng of total RNA was reverse transcribed with 200 U of M-MLV reverse transcriptase and 150 ng random hexamers according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). RT-PCR amplification was performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). The relative quantification method was used with 18S rRNA as the endogenous control.

Expression analyses of *HMGA2* and 18S *rRNA* were performed in triplicate in a total volume of 20 μl using 2 μl of each cDNA corresponding to 25 ng of total RNA. For the measurement of gene expression the following

Table 1. Patient demographics and clinical characteristics.

Disease	Type A Dissection, N=19	Ascending Aneurysms, N=26	Marfan Syndrome, N=2	Valve Replacement, N=4
Demographic				
Age (years)	59.6±16.4	59.3±13.8	31.5±6.4	54.0±9.2
Gender (male/female)	9m/10f	22m/4f	2m	4m
Body mass index	26.2±4.0	26.1±3.4	25.5±7.8	27.7±5.5
Aortic diameter (mm)	n.a.	53.3±7.1	70.0±14.1	38.0±1.6
Bicuspid aortic valve	N=2	N=14	0	N=3

Absolute values (± SD); n.a.: not available.

sequences were used: *HMGA2*: assay number Hs00171569, Applied Biosystems, with amplicon size of 65bp; 18S rRNA: forward primer: AATTTCACA TAGCCCACTTACATTTACA; reverse primer: TTGATTCTAATAATCCCATGCTTTGA; TaqMan (probe with amplicon size of 65 bp): 6-FAM-ACTGAAGAGTAATCAATCTA-MGB. Due to the higher expression of 18S rRNA the cDNA for these samples was diluted 1:10. The PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

RNA isolation, reverse transcription, and qRT-PCR for let-7d measurement

Aortic tissue (80 mg) was homogenized with a TissueLyser (Qiagen, Hilden, Germany) in 1000 μl of TRIzol® Reagent following the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). The RNA was resuspended in 80 μl of nuclease-free water.

Real-time RT PCR was performed in two steps using TaqMan® The MicroRNA assays (Applied Biosystems, Darmstadt, Germany). Twenty nanograms each of miRNA *let-7d* and endogenous control *RNU6B* were reverse transcribed with gene-specific stem-loop primers according to the manufacturer's protocol using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). In addition, 0.9 μg/μL whole-pooled fetal RNA (Stratagene, Karlsruhe, Germany) was used as an additional control. Real-time PCR was conducted with a 2x Universal Master Mix and 20x TaqMan® assay. MicroRNA assays were purchased as pre-optimized assays using the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Darmstadt, Germany). Each reaction was run in triplicate, containing 1.33 μl of cDNA in a final reaction volume of 20 μl. Data were analyzed using the 7300 system software (Applied Biosystems, Darmstadt, Germany) and the linear regression plot for ΔC_t analyses between *let-7d* and *RNU6B* was about 0.1, whereas the *RNU6B* expression was constant in each run by a threshold of 25.0±0.7.

Immunohistochemical analysis and fluorescence double labeling

Immunohistochemical analyses were performed for HMGA2, the EMT markers SNAI1 and Vimentin, TGFB1, the endothelial cell marker CD34 and MKI-67, a nuclear protein associated with cell proliferation. Formalin-fixed and paraffin-embedded tissue sections (4 μm) were deparaffinized, rehydrated and washed with PBS before immunoperoxidase staining. The slides were incubated overnight at 4°C in a humidified chamber with 1:20 rabbit anti-HMGA2 (sc-30223, Santa Cruz Biotechnology, Santa Cruz, California, USA), 1:30 rabbit anti-SNAI1 (sc-28199, Santa Cruz Biotechnology, Santa Cruz, California, USA), 1:200 rabbit anti-Vimentin (HPA001762, Sigma-Aldrich, Munich, Germany), 1:50 mouse anti-CD34 (NCL-END, Leica Biosystems, Newcastle, United Kingdom), 1:100 mouse anti-TGFB1 (ab27969, Abcam, Cambridge, United Kingdom), and 1:400 mouse anti-MKI-67 (M7240, Dako, Glostrup, Denmark). A biotinylated anti-rabbit or anti-mouse link was used as a secondary antibody (for 30 min). The slides were then incubated with an avidin-biotin enzyme label (Vector Laboratories, California, USA) for 30 min and developed with AEC peroxidase substrate (Camon Laborservice GmbH, Wiesbaden, Germany) for 10 min. Finally, the slides were counter-stained with hematoxylin.

For fluorescence double labeling antibodies against Vimentin (rabbit anti Vimentin, Sigma-Aldrich, Munich, Germany) and N-Cadherin (mouse anti N-Cadherin, Abcam, Cambridge, United Kingdom) were used in concentrations of 1:200 and 1:25 respectively. Secondary antibodies with Texas red (Vimentin) (Santa Cruz Biotechnology, Santa Cruz, California, USA) and FITC (N-Cadherin) (Sigma-Aldrich, Munich, Germany) were used. Nucleoli were stained with DAPI.

Statistics

Satistical analysis was performed using Excel/WinSTAT software (R. Fitch software, Bad Krozingen, Germany). Data are presented as mean ± SD. A two-sided Mann-Whitney U-test was performed when continuous data were considered and a two-sided Fischer's exact test was used when relative frequencies were compared. The association between different parameters was analyzed by Pearson's correlation coefficient. Significant differences were assumed at $P \leq 0.05$.

Results

Measurement of HMGA2 gene expression in aortic tissue using qRT-PCR

The levels of *HMGA2* mRNA were quantified using real-time quantitative PCR. Gene expression was measured in the aortic tissue of 19 patients with acute aortic dissection (AAD), 26 patients with thoracic aortic aneurysm (TAA) and two patients suffering from Marfan syndrome (MS); aortic valve tissue of four patients who did not suffer from dissection was also included. Figure 1 demonstrates the log of quantified *HMGA2* expression using the ΔC_t method.

The expression of *HMGA2* in non-dissection aortic disease samples (including two MS, four aortic valves, and 26 TAA) was moderate (Fig. 1A). Samples obtained from patients with TAA featured a mean level of 8.6±9.7-fold expression when compared to the calibrator sample. Samples of aortic tissue from patients with Marfan syndrome showed a mean expression level of 7.9±5.8 fold and mean expression in aortic valve tissue was 5.5±3.9 fold. Differences between these groups were assessed with the Mann-Whitney U-test and no

significant variation was detected. These groups were considered to feature similar *HMGA2* expression and were amalgamated in a group designated "without dissection".

The *HMGA2* expression ranged from 5.2 to 1212.4 fold (mean level = 193.1±272.8) in tissues of AAD patients (Fig 1B). The difference between the AAD samples and the "without dissection" group was highly significant in the Mann-Whitney U-test ($P=1.4*10^{-7}$) but differences between AAD and the non-dissection subgroups were also significant.

The existence of valve malformations, such as the bicuspid aortic valves (BAV), had no significant impact on *HMGA2* expression in TAA patients (BAV vs. TAV: P=0.47). The involvement of age, BMI and aortic diameter was also considered, but none showed a significant correlation with *HMGA2* expression in the different groups (data not shown).

The 19 cases of AAD were composed of 9 male and 10 female individuals. The difference in age between male and female individuals with AAD was found to be significant (mean age male vs female = 50.4±18.0 vs 67.8±11.0 years, P=0.03). The *HMGA2* expression was three-fold higher in female AAD patients compared with male AAD patients (296.0±337.0 vs. 79.0±110.0 fold, P=0.02), (Fig 2). However, *HMGA2* expression was still

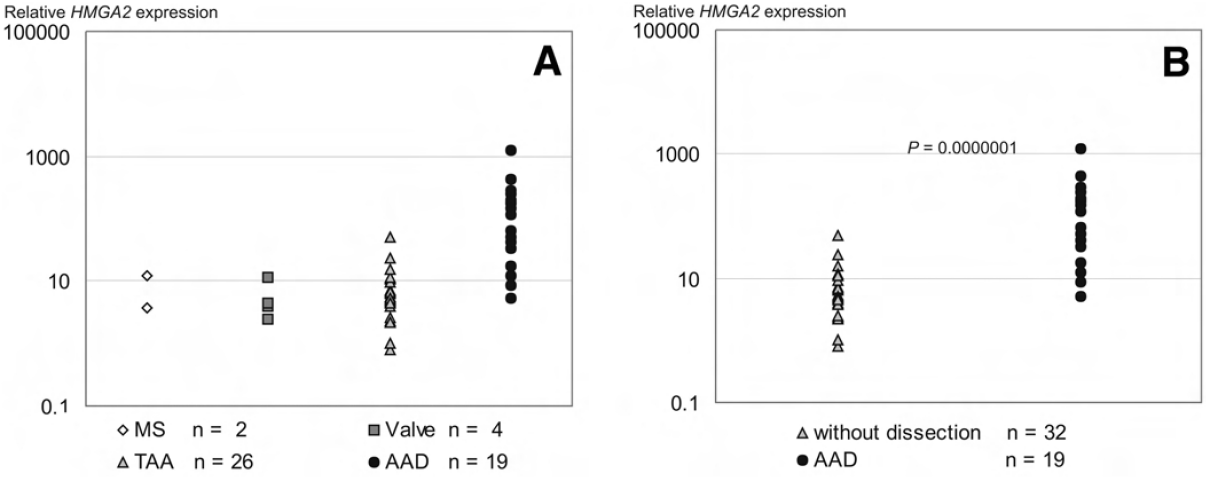

Fig. 1. Logarithmic data of *HMGA2* expression in tissue samples from patients with different aortic diseases. **A.** Comparison of *HMGA2* expression between patients with Marfan syndrome, tissue from aortic valves and patients with thoracic aortic aneurysm. **B.** *HMGA2* expression in different aortic tissues compared to AAD. Samples from Marfan syndrome patients, TAA patients and aortic valve tissue were grouped together and compared with samples obtained from acute aortic dissection, P=0.0000001.

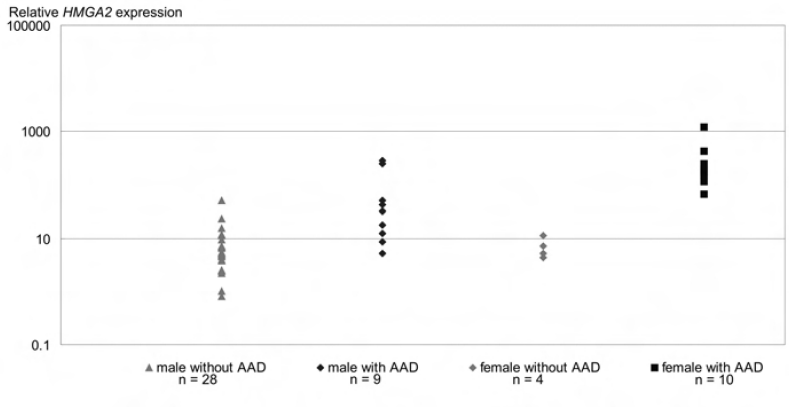

Fig. 2. Gender difference in HMGA2 expression of AAD patients. HMGA2 expression in AAD is three-fold higher in females compared with males (P=0.02). However, these differences in HMGA2 expression were nine-fold higher when we compared females/males with and without dissection (females with AAD vs. females without AAD: P=0.005, males with AAD vs. males without AAD: P=0.0007).

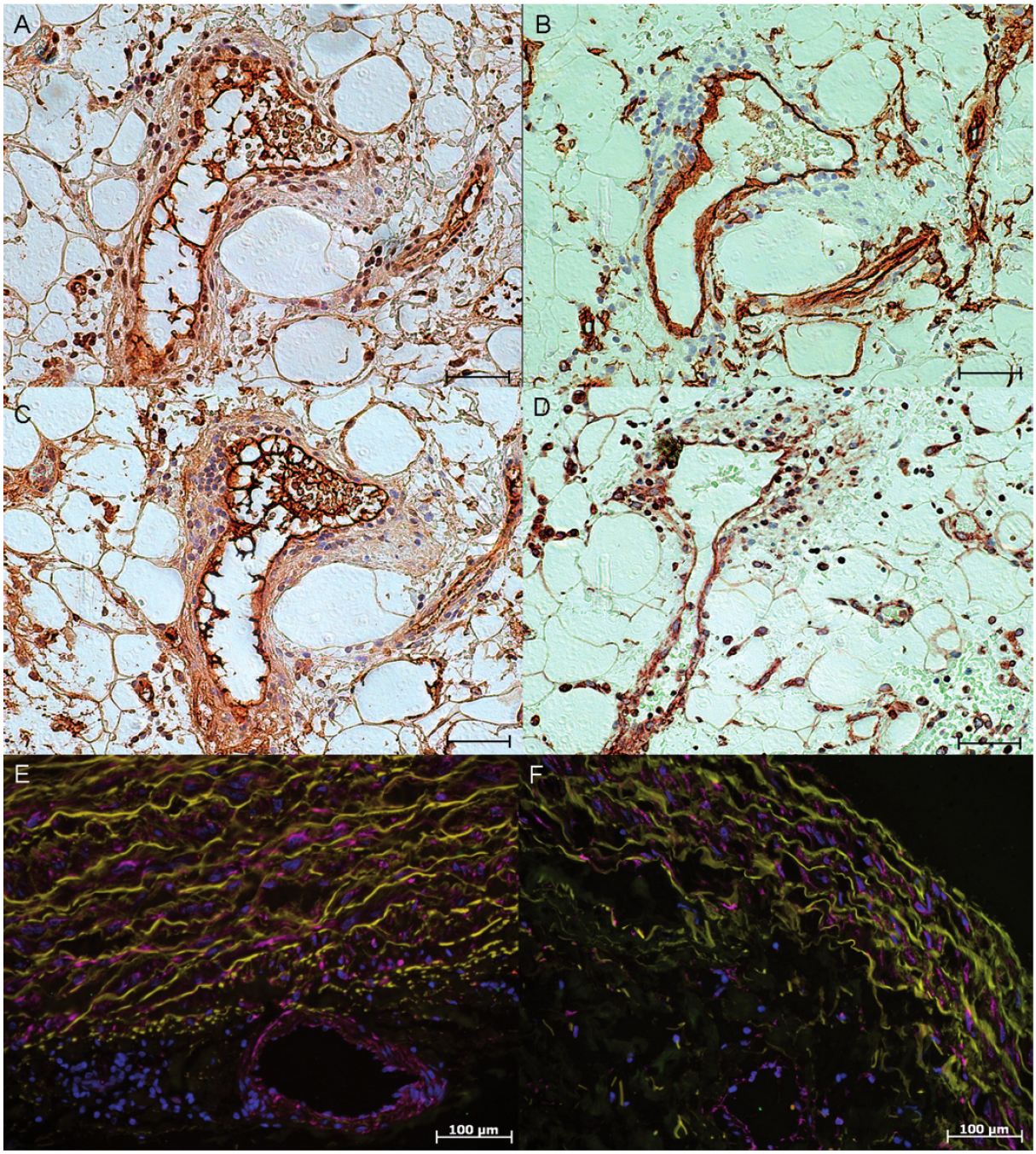

Fig. 3. Immunohistochemical staining and fluorescence double labeling of aortic tissue slides. **A.** HMGA2-positive cells in and around the *vasa vasorum* of an AAD case. **B.** Staining with CD34 reveals the inner vasa vasorum cells to be endothelium. **C.** Immunohistochemistry of the same *vasa vasorum* shows SNAI1 expression in the endothelial cells. **D.** Again the same vasa vasorum, this time the EMT marker Vimentin is found in and around the *vasa vasorum* endothelium. **E and F.** Fluorescence double labeling of two different cases. Purple staining shows the presence of Vimentin, which can also be detected in cells surrounding the *vasa vasorum.* Green/yellow staining indicates autofluorescence. Tissue slides in A, B, C and D were counter stained with hematoxylin. Scale bars: 100 μm.

nine-fold higher when we compared female or male AAD patients with their corresponding group without dissection (female with AAD vs. female without AAD: P=0.005, male with AAD vs. male without AAD: P=0.0007), (Fig 2).

Immunohistochemical analysis and fluorescence double labeling

Immunohistochemical analysis was performed to determine the localization of HMGA2, SNAI1, Vimentin, CD34, TGFB1 and MKI-67 proteins within the aorta. Tissue slides of AAD patients (n=11) and slides of tissue without AAD (n=11) were incubated with the antibodies (Fig. 3). For Vimentin staining tissue slides of 19 AAD patients and 10 patients without AAD were used. Fluorescence double labeling was carried out for the EMT markers Vimentin and N-Cadherin.

HMGA2-positive cells were found in the endothelium and abundantly in the cells surrounding the vasa vasorum (Fig. 3A). These cells were also proven to be endothelial by immunohistochemistry with the endothelial marker CD34 (Fig. 3B), but the EndMT marker SNAI1 was found here as well (Fig. 3C). Vimentin was used as a second marker for EndMT. It was also primarily located in the *vasa vasorum* endothelium and cells surrounding these endothelium (Fig. 3D). Fluorescence double labeling was only positive for Vimentin but negative for N-Cadherin (Fig. 3E,F). Again Vimentin was found in the endothelium of the *vasa vasorum*.

The TGFB1-positive cells were detected in all aortic layers but the staining was too diffuse for interpretation. No increased cell proliferation was detected using the

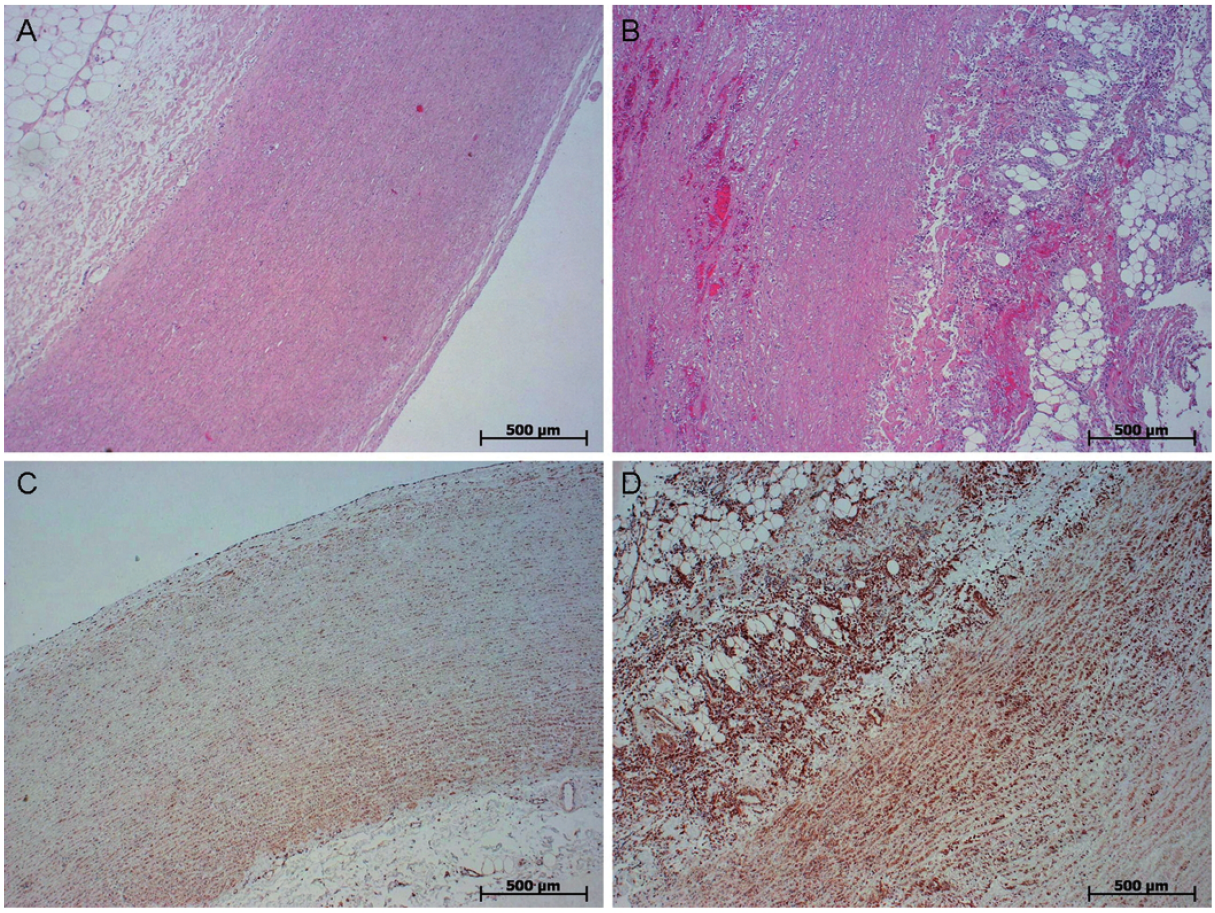

Fig. 4. Hematoxylin-eosin staining of AAD tissues and Vimentin immunostaining of comparable areas. **A and B.** H&E staining of AAD aortic tissue from two different patients. **C and D.** Detection of Vimentin in areas comparable to A and B. The vasa vasorum in the adventitia shows increased Vimentin abundance. Scale bars: 500 μm.

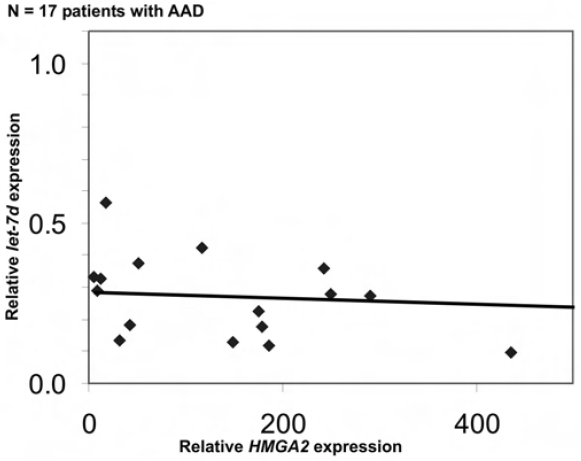

Fig. 5. Relative *HMGA2* and *let-7d* expression. Comparison of quantified relative *HMGA2* and *let-7d* expression of 17 patients with AAD revealed no significant differences. The correlation between the two sample sets (*let-7d* and *HMGA2* expression of 17 AAD patients) was very poor at R^2=0.04, P=0.86

human MKI-67 antibody (data not shown).

The HMGA2 and SNAI1 proteins were found in 7 out of 11 cases originating from AAD patients, whereas only 2 out of 11 cases from other aortic diseases, including aortic aneurysm, were positive (P=0.08). Vimentin was detected in 10 out of 19 AAD slides and in 2 out of 10 slides from control patients (P=0.1). The difference between these findings may improve in a greater sample number.

Figure 4 demonstrates Hematoxylin-eosin staining of AAD tissue and Vimentin immunostaining of the same area. Vimentin was observed in the *media* and *adventitia* and was most abundant in the *vasa vasorum*.

Detection of let-7d in aortic tissue of AAD

The level of the *let-7* variant *d* was quantified in 17 AAD tissue samples to study whether *HMGA2* expression in AAD is regulated by this miRNA. Figure 5 compares the *let-7d* expression calculated by the ΔC_t method with the quantified *HMGA2* expression in the same samples. The correlation between the two sample sets was very poor at R^2=0.045 and P=0.86, indicating that there is no relationship between *HMGA2* gene expression in AAD tissue and *let-7d* (Fig 5).

Discussion

To the best of our knowledge, this is the first report of an alteration of *high mobility group AT-hook 2* (HMGA2) levels in aortic dissection. In this study we present evidence that the *HMGA2* gene is significantly upregulated in patients with type A aortic dissection when compared to other aortic diseases such as thoracic aortic aneurysm. The difference of *HMGA2* expression between the AAD samples and all the other investigated tissues "without dissection" was highly significant in the Mann-Whitney U-test at $P=1.4*10^{-7}$.

We investigated in this study a whole of 51 patients (19 AAD, 26 TAA, two Marfan syndrome and four aortic valve) and acknowledge that the 19 studied cases of acute aortic dissection type A were composed of 9 male and 10 female individuals, which contradicts data in the literature that reports acute aortic dissection as being 2.8 times more common in males than in females (Grundmann et al., 2006).

We found the female AAD patients to be of significantly higher age than their male counterparts (67.8±11.0 vs 50.4±18.0 years, *P*=0.03). This might be attributed to the functions of estrogens, which protect females from cardiovascular disease. Depletion of estrogen after the menopause leads to an increase of cardiovascular diseases in females of advanced age and might explain the fact that all female dissection cases in this study were 51 years old and above (Tan and Pu, 2004). Female patients featured also a significantly higher *HMGA2* expression (296.0±337.0 vs. 79.0±110.0 fold, *P*=0.02), which might relate to their higher age.

In the immunohistochemical analysis we determined the expression site of HMGA2 in AAD tissue. This protein is mainly found in cells surrounding the *vasa vasorum*. The *vasa vasorum* penetrates the *tunica media and tunica adventitia* for nutrition of the aortic layers. Immunohistochemistry of the endothelial marker CD34 revealed that the *HMGA2*-positive cells were endothelial, while the EMT marker protein SNAI1 was also found mainly in these cells. Additionally, Vimentin, member of the intermediate filament family, whose expression is upregulated during the transition of endothelial cells to mesenchymal cells, was detected in the *vasa vasorum* endothelium of AAD patients by immunohistochemistry and fluorescence double labeling. Expression of N-Cadherin, a secondary EMT marker, could not be found in fluorescence double labeling. N-Cadherin should be detectable at least in vascular smooth muscle cells as reported by various studies (Moiseeva, 2001). However the N-Cadherin antibody was proven to be functional in control tissue, so the meaning of this observation remains unclear. The presence of the EMT markers SNAI1 and Vimentin in endothelial cells indicates a possible endothelial-to-mesenchymal transition (EndMT), a form of EMT of the *vasa vasorum* endothelial cells. The absence of MKI-67 in the endothelium of the *vasa vasorum* suggests that no increased cell proliferation was detectable. This means that *HMGA2*, SNAI1 and Vimentin are expressed independently of cell proliferation. Aortic dissections arise from an entry tear in the aortic *tunica intima* or by intramural hematoma from rhexis of aortic *vasa vasorum* (Nienaber and Sievers, 2002; Tsai et al., 2005). EndMT in the *vasa vasorum* could lead to a loss of endothelial cells and a weakening of the endothelium, which would

render the *vasa vasorum* more prone to rupture.

The *Let-7d* microRNA was quantified in AAD patients and compared to *HMGA2* mRNA expression. Quantification at the mRNA level seems reasonable since human microRNA, unlike the *C. elegans* or *Drosophila counterparts*, tends to degrade its target mRNA completely (Smalheiser and Torvik, 2004). Moreover, a negative correlation between *let-7a* miRNA and *HMGA2* has already been found in gastric cancer cell lines (Motoyama et al., 2008). In our case no significant correlation was found between *HMGA2 and let-7d,* which suggests that *HMGA2* is expressed in a *let-7d*-independent manner in these patients. A possible explanation for this is the regulation of *HMGA2* by another *let-7* variant. It is also reasonable to assume that *let-7d*-independent *HMGA2* expression occurs only in patients with AAD, and that dysfunction of the *let-7* regulatory effect might be the reason for the overexpression of *HMGA2* in AAD. Alternatively, in these cases upregulation may occur at the transcriptional level.

In Marfan syndrome *Fibrillin-1*, a gene coding for a microfibrillar protein of the extracellular matrix, was found to be mutated (Dietz et al., 1991; Dietz and Pyeritz, 1995). Microfibrils composed of *Fibrillin-1* associate with the latent TGFB binding protein (LTBP), which controls the availability of TGFB in the extracellular matrix. Mutation of *Fibrillin-1* leads to dysregulation of the TGFB pathway and is therefore considered to be a critical event in the formation of AAD in Marfan syndrome (Neptune et al., 2003; Robinson et al., 2006). In a previous survey we found TGFB1 to be among the regulators of the differentially expressed genes in AAD (Mohamed et al., 2009). In this study we investigated a further 19 patients with AAD. They presented neither a clinical manifestation of Marfan syndrome nor mutation-related alterations in the *Fibrillin-1* gene. Of note, the TGFB1 protein was detected in massive amounts in almost all of the cells, including activated T cells and macrophages, and in all three layers of the aorta. However, we were not able to quantify the TGFB1 level due to its diffuse appearance. The signaling of TGFB has been implicated in pathways leading to enhanced extracellular matrix degradation and the production of various matrix metalloproteinase species (Kim et al., 2004). Since *HMGA2* acts downstream of TGFB in the EMT, the changing of the *vasa vasorum* by the transition of endothelial cells might be caused by TGFB dysregulation independent of a *Fibrillin-1* mutation.

Altogether these results suggest a possible remodeling of the *vasa vasorum* endothelium by the HMGA2-induced transition of endothelial cells to mesenchymal cells.

These mechanisms could lead to an impairment of the *vasa vasorum* and to the formation of intramural hematomas which are considered to be a predecessor of dissection (Nienaber and Sievers, 2002).

Alternatively the EndMT might be a repair mechanism as a consequence of tissue injury. Recent studies suggest a re-expression of *HMGA2* in pathophysiological adult tissues as a response to biochemical stress (Monzen et al., 2008).

Our results revealed for the first time the overexpression of the *HMGA2* gene in patients with AAD. This expression seems to be independent of regulatory miRNA *let-7d* and occurs mainly in the *vasa vasorum*. Since the EMT-causing transcription factor SNAI1 was expressed together with the EMT marker Vimentin in the endothelium of the *vasa vasorum,* the EndMT of these cells, induced by elevated *HMGA2* levels, seems possible. We assumed that upregulation of *HMGA2* seems to cause remodeling of the aortic wall by EndMT or it is a consequence of the aortic injury and repair activity following the acute events.

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Conflict of interest. None declared.

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3.2 Publication II: Locally different endothelial nitric oxide synthase protein levels in ascending aortic aneurysms of bicuspid and tricuspid aortic valve

The presence of a bicuspid aortic valve (BAV) harbours an increased risk for the development of aortic aneurysms and dissections[41, 43]. The pathogenic mechanisms behind this relationship are still unclear but two theories exist, one favouring a genetic defect, the other increased shear stress on the aortic wall[58, 59]. In a study by Aicher et al. a significantly lower expression of the endothelial nitric oxide synthase (eNOS) was found in the aorta of BAV subjects. Furthermore, there was a significant correlation between the amount of eNOS protein and the aortic diameter and it was concluded that eNOS may play a role in BAV associated aortopathy[63].

The eNOS is an enzyme which catalyses the conversion of L-arginin to L-citrullin and nitric oxide (NO), a signalling molecule which regulates vascular homeostasis[110]. Activity and expression of the enzyme are controlled by hemodynamic stimulation of the endothelial cells through fluid shear stress[64, 65, 111, 112].

This study focuses on the assumption that irregular shear stress caused by the malformed aortic valve can lead to spatial dysregulation of eNOS within the aorta and therefore cause changes in the aortic environment, which in turn could give rise to aortic aneurysms. To test this hypothesis the eNOS protein amount was determined in four different aortic areas of 14 BAV and 5 TAV aneurysm cases.

Additionally the protein levels of 35 apoptosis-related proteins were quantified after inhibition of NO synthesis, since it is documented that NO can exhibit an anti-apoptotic effect and that apoptosis of vascular smooth muscle cells acts as a mechanism of aneurysm formation in BAV[113-115].

The eNOS protein expression showed a different spatial distribution in BAV associated aneurysms than in TAV aneurysms, with a higher eNOS amount in the BAV concavity than in its TAV counterpart but a significantly reduced eNOS level in the BAV proximal aorta compared to the TAV proximal aorta. These results suggest that eNOS is indeed spatially dysregulated in the BAV aorta, probably due to the change in shear stress distribution.

Inhibition of NO synthesis in vascular smooth muscle cells (VSMCs) and human aortic endothelial cells (HAECs) lead to little differences in the protein expression of

the 35 apoptosis-related proteins. Only the cytosolic amount of the pro-apoptotic serine protease HTRA2/Omi was significantly increased after NO synthesis inhibition in VSMCs ($p = 0.038$) but Cytochrome c levels in the cytosol of HAECs were also higher after the treatment, albeit not significant ($p = 0.069$). Both proteins are released from mitochondria into the cytosol during the intrinsic apoptosis pathway[116, 117]. These data give further insight into the way NO confers its anti-apoptopic effects.

-II-

Locally different endothelial nitric oxide synthase protein levels in ascending aortic aneurysms of bicuspid and tricuspid aortic valve

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Own contribution:

- Extraction of the cytosolic protein fraction of aortic cells
- Quantification of eNOS protein levels in aortic tissue
- Inhibition of NOS in human cells
- Quantification of 35 apoptosis-related proteins with proteome profiler arrays
- Statistical analysis
- Co-writing of the manuscript

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Research Article

Locally Different Endothelial Nitric Oxide Synthase Protein Levels in Ascending Aortic Aneurysms of Bicuspid and Tricuspid Aortic Valve

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Aims. Dysregulated expression of the endothelial nitric oxide synthase (eNOS) is observed in aortic aneurysms associated with bicuspid aortic valve (BAV). We determined eNOS protein levels in various areas in ascending aortic aneurysms. *Methods and Results.* Aneurysmal specimens were collected from 19 patients, 14 with BAV and 5 with tricuspid aortic valve (TAV). ENOS protein levels were measured in the outer curve (convexity), the opposite side (concavity), the distal and above the sinotubular junction (proximal) aneurysm. Cultured aortic cells were treated with NO synthesis inhibitor L-NAME and the amounts of 35 apoptosis-related proteins were determined. In patients with BAV, eNOS levels were significantly lower in the proximal aorta than in the concavity and distal aorta. ENOS protein levels were also lower in the convexity than in the concavity. While the convexity and distal aorta showed similar eNOS protein levels in BAV and TAV patients, levels were higher in TAV proximal aorta. Inhibition of NO synthesis in aneurysmal aortic cells by L-NAME led to a cytosolic increase in the levels of mitochondrial serine protease HTRA2/Omi. *Conclusion.* ENOS protein levels were varied at different areas of the aneurysmal aorta. The dysregulation of nitric oxide can lead to an increase in proapoptotic HTRA2/Omi.

1. Introduction

Bicuspid aortic valve (BAV) is a common congenital cardiac defect having a prevalence of 0.9% to 2%. It is associated with stenosis, insufficiency, and ascending aortic aneurysms [1]. The formation of ascending aortic aneurysms in BAV patients seems to be linked to apoptosis of medial vascular smooth muscle cells (VSMCs). Apoptosis was found to be more frequent in the medial tissue of dilated aortas and cultured VSMCs derived from aneurysmatic aortas. Additionally cultured VSMCs derived from BAV dilated aortas showed higher apoptosis rates than VSMCs from control patients [2, 3].

The endothelial nitric oxide synthase (eNOS) is associated with the development of BAV, and eNOS-produced nitric oxide (NO) is also believed to play a role in aneurysm formation. In 2000 Lee and colleagues showed that eNOS-deficient mice were predisposed to develop a BAV, whereas Kuhlencordt et al. found a higher incidence of aortic aneurysm in eNOS/Apolipoprotein E double-knockout mice [4, 5]. A study by Aicher et al. presented a significant decrease in eNOS protein amount in BAV aortic tissue compared to TAV aortic tissue [6]. Expression and activity of eNOS in aortic endothelial cells are controlled by hemodynamic wall shear stress [7, 8]. Recent publications have indicated that aortic wall shear stress differs locally between BAV and control patients when examined via magnetic resonance imaging (MRI) [9, 10]. Furthermore we provided evidence that VSMCs show a different apoptotic behavior in the concave versus the convex side of the dilated aorta. Inhibition

Table 1: Patient characteristics.

Characteristics	BAV ($n = 14$)	TAV ($n = 5$)	P-value
Age (years)	51.9 ± 12.2	59.4 ± 8.7	0.257
Sex (f/m)	3/11	1/4	0.5
BMI (kg/m^2)	27.5 ± 4.2	28.6 ± 8.2	0.333
Maximum sinus diameter (mm)	38.3 ± 5.1	53.0*	—
STJ diameter (mm)	36.2 ± 4.1	53.0*	—
Tubular diameter (mm)	51.5 ± 4.6	55.4 ± 7.3	0.444
Arch diameter (mm)	39.5 ± 7.3	n.a.	—
Aortic stenosis	6/14 (42.9%)	0/5 (0%)	0.1
Aortic insufficiency	3/14 (21.4%)	5/5 (100%)	0.005
Combined stenosis/insufficiency	5/14 (35.7%)	0/5 (0%)	0.2

BAV: bicuspid aortic valve; BMI: body mass index; f: female; m: male; n.a.: not available; STJ: sinotubular junction; TAV: tricuspid aortic valve; *only data from two patients were available, and therefore statistics were omitted.

of caspase-3 was shown to protect cultured cells derived from the concavity of the aorta more significantly than those from the convexity [11]. Taken together, these results suggest a locally different behavior of medial cells in BAV aortic tissue than in TAV aortic tissue [12]. This is particularly interesting because it is known that eNOS-derived NO can inhibit caspases via S-nitrosylation [13], which might explain the observed differences in apoptosis between the different aortic areas. Therefore, we determined eNOS protein levels at different aortic areas of aneurysmal BAV and TAV patients. We also determined the effects of NO-synthesis inhibition on apoptosis of primary human aortic endothelial cells (HAECs) and human aortic VSMCs taken from tissue of a BAV patient.

2. Methods

2.1. Sample Collection. The investigation conforms with the principles outlined in the Declaration of Helsinki for use of human tissue. The study protocol was approved by the institutional ethics committee, and written informed consent was obtained from all patients. Specimens of aortic wall of 14 BAV and 5 TAV aneurysmal patients were collected during surgery (patient characteristics are shown in Table 1). The tissue was taken from 4 distinct areas of the aorta, the concave side of the aorta (inner curves), the convex side of the aorta (outer curves, the right anterolateral aspect), the distal aneurysm (under the aortic arch), and the proximal aneurysm (above the sinotubular junction), and immediately placed in liquid nitrogen. Cell lysate was prepared using the Bio-Plex Cell Lysis Kit according to the manufacturer's instructions (BIO-Rad, Hamburg, Germany). Whole protein concentration in the cell lysate was measured using the BCA protein assay (Thermo Scientific, Rockford, USA).

2.2. Western Blot Analysis. The wells of NuPAGE 10% Bis-Tris gels (Invitrogen, Darmstadt, Germany) were loaded with 20 μg of cell lysate, and a denaturising SDS-PAGE was run using the Xcell SureLock Mini-cell (Invitrogen, Darmstadt, Germany) and appropriate buffers. The separated proteins were transferred to a nitrocellulose membrane, which was blocked with TBS-T containing 5% (v/w) BSA at 4°C overnight. The membrane was washed thrice in TBS-T and incubated with primary antibody diluted in TBS-T containing 5% BSA (v/w) for 1 h. Then, the washing step was repeated, and the membrane was treated with secondary antibody in TBS-T containing 5% BSA (v/w) for 1 h. NOS3 rabbit polyclonal antibody (sc-654) was used together with bovine anti-rabbit IgG-AP (sc-2372) secondary antibody (Santa Cruz Biotec, Heidelberg, Germany) to detect eNOS protein. The membrane was washed thrice with TBS-T and thrice with TBS before protein bands were visualized by incubation of the membrane in staining solution made of 300 μL NBT/BCIP stock solution (Roche, Mannheim, Germany) and 15 mL substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$).

Band intensity was analyzed using the imageJ software. To compare band intensity between different blots, a calibrator sample was run on every blot.

Both beta-actin (Novus Biologicals, Heford, Germany) and GAPDH (Santa Cruz Biotec, Heidelberg, Germany) were tested as loading control. Both controls displayed a high fluctuation in band intensity. Therefore the initial loading amount was used to normalize eNOS band intensity.

2.3. Cell Culture. Vascular smooth muscle cells (VSMCs) were taken from the *tunica media* of a BAV patient. For cell culture this sample was minced and treated with 0.26% collagenase (250 U/mL, Serva, Heidelberg, Germany) at 37°C for about 3-4 h. After centrifugation, the pellet was resuspended in culture medium (TC199 with Earle's salts supplemented with 20% fetal bovine serum, 200 U/mL penicillin, 200 μg/mL streptomycin) and incubated at 37°C in 5% CO_2 air. After attachment of the tissue pieces 5 mL fresh medium were added. The monolayer culture was passaged by standard trypsine dispersion and resuspended in TC199 culture medium.

Primary human aortic endothelial cells (HAECs) were purchased from Lonza and cultured in endothelial cell growth medium-2 (Lonza, Cologne, Germany).

2.4. Transfection and Characterization of Human Aortic VSMCs. Human aortic VSMCs obtained from a first passage monolayer culture were plated in 25 cm^2 flasks and allowed to attach and grow for 48 h until the culture reached approximately 80% confluency. The subconfluent muscle cell culture was then transfected with 5 μg of pSV40-dN-plasmid DNA as described previously [14, 15] using Effectene kit (Qiagen; Hilden, Germany). Culture supernatant containing transfecting solution was removed after 1 h. Cells were washed with PBS and cultured in TC199 medium containing 20% FCS and 2% (v/v) antibiotic solution. When cultures showed focus formation, foci were isolated and subcultured in TC199 culture medium containing 20% FCS and 2% (v/v) antibiotic solution. The population doubling time was measured as described previously [14].

For cell characterization, cytosolic proteins were extracted and analyzed in western blot. In short cells were washed with 1x PBS (4°C), and 500 μL of cell lysis buffer (150 mM NaCl, 50 mM TrisHCl, 0.5% deoxycholic acid, 1% NP-40) containing 1x complete mini protease inhibitor (Roche, Mannheim, Germany) were added. A cell scraper was used to remove the VSMCs from the cell culture flask, and the suspension was incubated on a shaker on ice for 35 min. Subsequently the cell lysate was centrifuged at 14,200 ×g and 4°C for 20 min to collect the cytosolic protein fraction in the supernatant. The whole protein amount was determined using the BCA protein assay (Thermo Scientific, Rockford, USA). Western blot analysis was performed as described above. A smooth muscle alpha-actin primary antibody (Mab 1420; R&D Systems, Wiesbaden-Nordenstadt, Germany) was used as a smooth muscle cell marker, and a von Willebrand factor primary antibody (sc-53466; Santa Cruz, Heidelberg. Germany) was used as marker for endothelium. Since cells did contain smooth muscle alpha-actin but not von Willebrand factor (data not shown) they were considered as vascular smooth muscle cells (VSMCs).

2.5. L-NAME Treatment. VSMCs and HAECs were grown to 90% confluency and then incubated in serum-free medium for 24 h. Afterwards cells were treated with 1 mM of NO synthase inhibitor L-NAME for one hour. The cytosolic protein fraction was extracted as described above.

2.6. Quantification of Apoptosis-Related Proteins. For quantification of 35 apoptosis-related proteins after L-NAME treatment, 200 μg of cytosolic protein were used in conjunction with the proteome profiler array (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. In short, the arrays were blocked for 1 h and then incubated with 200 μg protein lysate at 4°C over night. Afterwards the arrays were incubated with biotinylated detection antibody cocktail (1 : 1000, R&D Systems, Wiesbaden-Nordenstadt, Germany) for one hour, and antibiotin alkaline phosphatase conjugated secondary antibody (1 : 10,000, Sigma-aldrich, Munich, Germany) for 30 minutes. Protein spots were visualized with solution made of 300 μL NBT/BCIP stock solution (Roche, Mannheim, Germany) and 15 mL substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$). Spot intensity was quantified using the imagej software. The spots of the positive control were used as calibrator. Data were taken from three independent experiments with two replicates each.

2.7. Statistical Analysis. Statistical analysis was done with SPSS and Excel/WinSTAT, and all data are displayed as absolute numbers and relative percentages or as mean ± standard deviation. Differences in eNOS protein expression were tested with two-sided Mann—Whitney U-test for independent variables (comparison between BAV and TAV areas) or with Wilcoxon signed-rank test for dependent variables (comparison between different BAV or TAV areas). Differences in apoptosis-related proteins after L-NAME treatment were analysed with repeated measurement two-way ANOVA to account for the two replicates on each apoptosis array. Relative frequencies were compared using the fisher exact test. Significant differences were assumed at $P < 0.05$.

3. Results

3.1. Determination of eNOS Protein Levels in Different Aortic Areas. Aortic tissue was taken during aneurysmal replacement from 14 patients with BAV and from 5 patients with TAV. Patients with BAV tended to be younger, having a low BMI, smaller aortic diameter with a higher percentage of aortic stenosis and combined stenosis/insufficiency but a significantly lower percentage of aortic insufficiency (21.4% versus 100%, $P = 0.005$). However, none of these characteristics differed significantly along with gender distribution and the percentage of aortic insufficiency (see Table 1). So we assumed these patient groups to be comparable.

Protein levels of eNOS were quantified in the concavity, distal aneurysm, convexity, and proximal aneurysm of these BAV and TAV patients (Figure 1).

In TAV patients, eNOS protein level was least in the concavity, at a medium level in the distal aorta and convex side and highest in the proximal aneurysmal aorta. Nonetheless there was no significant difference in eNOS amounts between the TAV aneurysmal aortic areas. BAV patients on the other hand featured the highest eNOS protein levels in the concavity, while distal aneurysmal aorta and convexity showed medium levels and the proximal aneurysmal aorta showed low eNOS protein level. Several of these differences proved to be significant (concavity versus convexity, 93.14 ± 48.47 versus 59.67 ± 46.03% of calibrator, $P = 0.04$; concavity versus proximal aorta, 93.14 ± 48.47 versus 43.00 ± 53.38% of calibrator, $P = 0.02$; distal versus proximal aorta, 61.2 ± 31.8 versus 43.00 ± 53.38% of calibrator, $P = 0.03$) (Figure 1(b)).

The eNOS protein amount in the TAV concave side of the aorta was lower than that in the BAV concavity but this difference was not significant (49.57 ± 23.6 versus 93.14 ± 48.47% of calibrator, $P = 0.07$). In the distal aorta and the convex aortic side there was little difference between TAV and BAV eNOS protein amounts (distal aorta: 63.23 ± 31.99 versus 61.2 ± 31.8% of calibrator, $P = 0.96$; convexity: 61.63 ± 35.38 versus 59.67 ± 46.03% of calibrator, $P = 0.75$). However, in the proximal aorta, eNOS levels were significantly higher in TAV patients than BAV patients (105.79 ± 75.11 versus 43.00 ± 53.38% of calibrator, $P = 0.04$) (Figure 1(c)).

3.2. Quantification of Apoptosis-Related Proteins after Treatment of VSMCs and HAECs with 1 mM L-NAME. To determine the effect a dysregulation of eNOS would have on aortic cell apoptosis, we treated VSMCs originating from aortic tissue and primary HAECs with 1 mM of the specific NO-synthase inhibitor L-NAME for one hour. Afterwards 35 apoptosis-related proteins were quantified using a proteome profiler array for human apoptosis. In VSMCs most apoptotic proteins increased in abundance, although only HTRA2/Omi levels were significantly elevated after L-NAME treatment ($P = 0.04$, Figure 2).

Treatment with L-NAME resulted in little difference in Bax, Bcl-2, and cleaved caspase-3 concentration. The cytosolic amount of cytochrome c was slightly elevated

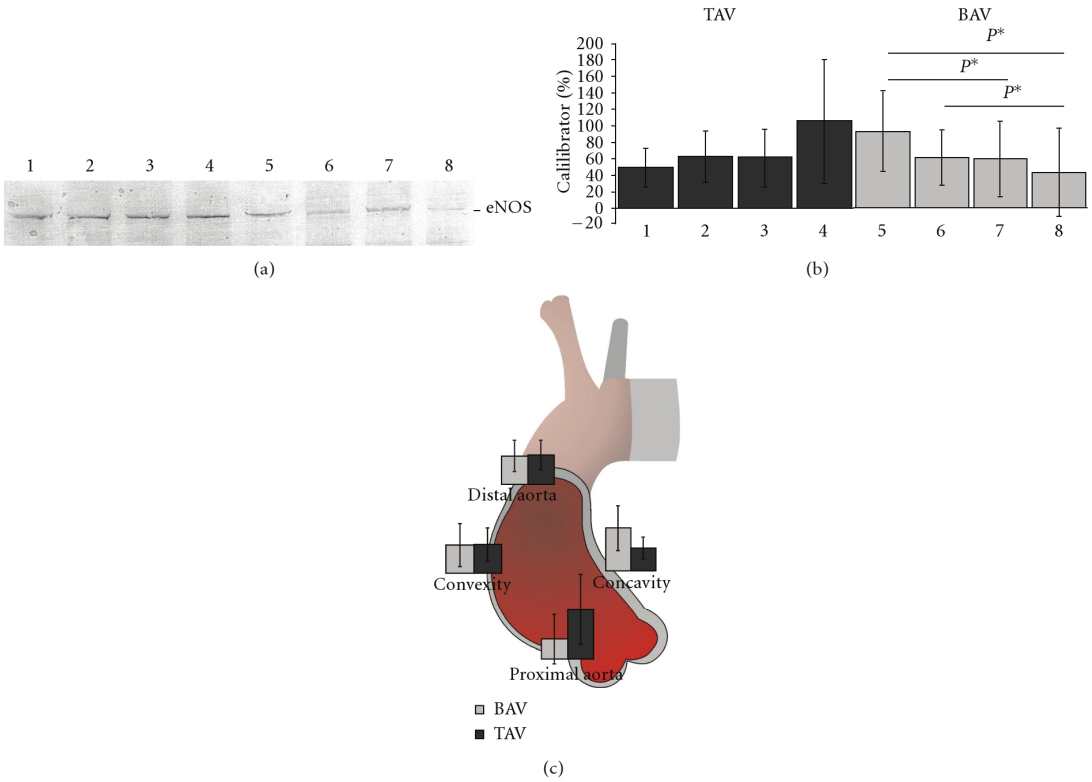

Figure 1: (a) Representative western blot of pooled cell lysate from 4 TAV and 4 BAV patients. 1: TAV concavity; 2: TAV distal aneurysm; 3: TAV convexity; 4: TAV proximal aneurysm; 5: BAV concavity; 6: BAV distal aneurysm; 7: BAV convexity; 8: BAV proximal aneurysm. (b) The eNOS protein level ± SD in different aortic areas was determined by densitometry. 1: TAV concavity; 2: TAV distal aneurysm; 3: TAV convexity; 4: TAV proximal aneurysm; 5: BAV concavity; 6: BAV distal aneurysm; 7: BAV convexity; 8: BAV proximal aneurysm; P^*: $P < 0.05$; TAV: $n = 5$; BAV: $n = 14$. (c) eNOS protein levels in different areas of TAV and BAV aneurysmal aorta. Mean levels of eNOS protein amounts ± SD are given for each aortic area. Differences between eNOS protein levels of TAV ($n = 5$) and BAV ($n = 14$) in the proximal aneurysmal aorta are significant with $P = 0.04$.

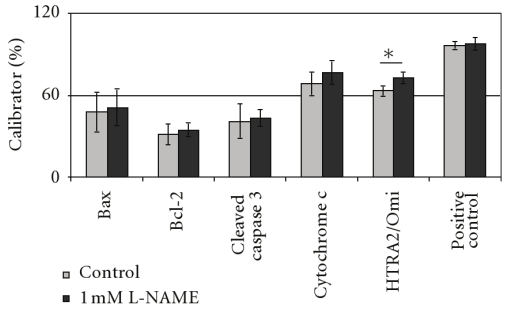

Figure 2: Change in concentration of apoptosis-related proteins after treatment with L-NAME. Bars display the change in concentration of Bax, Bcl-2, cleaved caspase 3, cytochrome c, HTRA2/Omi, and the positive control after treatment of VSMCs with 1 mM L-NAME for 1 h. *: $P = 0.04$.

(68.46±8.61 versus 76.39±8.48% of calibrator) although this difference was not significant ($P = 0.336$). Cytosolic protein levels of HTRA2/Omi on the other hand were significantly increased after inhibition of NO synthesis (63.1 ± 3.8 versus 72.6 ± 4.2% of calibrator, $P = 0.04$). For changes in other apoptosis-related proteins see Tables 2 and 3. HTRA2/Omi is a mitochondrial serine protease, which is released into the cytosol by proapoptotic members of the Bcl-2 family [15]. Here it contributes to apoptosis by cleavage of members of the inhibitor of apoptosis (IAP) family, which otherwise function as direct inhibitors of caspase-3, -7, and -9 [16, 17]. Therefore these results indicate some form of early apoptosis in VSMCs after L-NAME treatment.

In HAECs no significant changes in apoptosis-related proteins could be observed after L-NAME treatment (Figure 3, see also Tables 2 and 3).

Bax, Bcl-2, cleaved caspase-3, and HTRA2/Omi did not display any changes in cytosolic protein levels. Cytosolic appearance of cytochrome c increased after L-NAME treatment (62.37 ± 11.07 versus 84.29 ± 6.92% of calibrator) but this difference was not significant ($P = 0.069$).

Table 2: Protein levels of 35 apoptosis-related proteins after treatment of VSMCs with 1 mM L-NAME.

Protein	Control average	1 mM L-NAME average	Change	P
Bad	50.59 ± 8.08	54.73 ± 9.86	4.15	0.637
Bax	47.65 ± 14.26	50.98 ± 13.43	3.32	0.805
Bcl-2	31.37 ± 7.47	34.32 ± 5.04	2.95	0.633
Bcl-x	17.62 ± 4.92	19.02 ±6.20	1.40	0.780
Pro-Caspase 3	107.21 ± 20.89	109.96 ± 19.89	2.75	0.889
Cleaved Caspase 3	40.89 ± 12.79	43.35 ± 6.22	2.46	0.799
Catalase	93.40 ± 24.75	97.04 ± 25.26	3.63	0.880
clAP-1	39.80 ± 20.28	45.09 ± 20.87	5.28	0.792
clAP-2	16.72 ± 4.71	18.86 ± 5.27	2.15	0.630
Claspin	40.49 ± 6.13	43.37 ± 6.39	2.89	0.618
Clusterin	22.70 ± 6.30	27.93 ± 7.19	5.23	0.437
Cytochrome c	68.46 ± 8.61	76.39 ± 8.48	7.93	0.336
TRAIL R1/DR4	40.74 ± 6.48	46.36 ± 3.04	5.62	0.241
TRAIL R2/DR5	66.06 ± 16.01	77.34 ± 6.60	11.28	0.363
FADD	64.20 ± 13.56	71.59 ± 4.95	7.39	0.467
Fas/TNFSF6	66.16 ± 23.27	68.26 ± 13.45	2.10	0.904
HIF-1a	29.40 ± 16.62	41.97 ± 15.61	12.57	0.436
HO-1/HMOX1/HSP32	27.06 ± 14.68	35.64 ± 12.58	8.58	0.526
HO-2/HMOX2	38.85 ± 14.23	49.90 ± 18.08	11.04	0.497
HSP27	33.21 ± 7.16	38.64 ± 9.88	5.43	0.528
HSP60	65.84 ± 11.85	70.83 ± 7.05	4.99	0.545
HSP70	66.75 ± 13.76	64.40 ± 5.59	−2.35	0.801
HTRA2/Omi	**63.09 ± 3.83**	**72.57 ± 4.18**	**9.48**	**0.038***
Livin	14.08 ± 2.52	18.83 ± 5.12	4.75	0.223
PON2	28.81 ± 5.92	31.30 ± 5.53	2.49	0.651
p21/CIP1/CDNK1A	27.93 ± 9.01	38.74 ± 9.16	10.81	0.259
p27/Kip1	16.75 ± 6.04	21.34 ± 2.89	4.59	0.345
Phospho-p53 (S15)	82.25 ± 19.34	88.18 ± 11.03	5.93	0.696
Phospho-p53 (S46)	64.83 ± 18.94	71.42 ± 20.27	6.59	0.729
Phospho-p53 (S392)	64.02 ± 28.92	75.33 ± 22.81	11.31	0.659
Phospho-Rad17 (S635)	11.71 ± 2.21	18.72 ± 5.65	7.01	0.129
SMAC/Diablo	56.47 ± 11.12	59.84 ± 9.98	3.36	0.742
Survivin	57.52 ± 23.63	61.96 ± 19.33	4.44	0.831
TNF RI/TNFRSF1A	18.18 ± 4.27	22.05 ± 1.64	3.87	0.210
XIAP	49.96 ± 5.39	56.28 ± 3.45	6.32	0.149

All values are in percent of the calibrator (positive control). The change is given in percent points of the calibrator. The data were acquired in three independent experiments. *: $P < 0.05$.

4. Discussion

The bicuspid aortic valve (BAV) is often associated with ascending aortic aneurysms. Ascending aortic aneurysms are marked by a pathologic dilatation of the aorta to the at least 1.5-fold diameter. The enzyme endothelial nitric oxide synthase (eNOS) catalyses the conversion of L-arginine to L-citrulline and nitric oxide (NO), which serves as a signaling molecule in the cardiovascular system [18, 19]. NO is known to mediate several vasoprotective properties, like inhibition of vascular smooth muscle cells (VSMCs) and proliferation and maintaining of endothelial function [20, 21].

Evidence for a connection between eNOS and BAV was given when Aicher et al. compared eNOS protein expression in patients with TAV and patients with BAV. They showed a downregulation of eNOS in the proximal aorta of BAV patients compared to TAV patients [6]. Our results demonstrate a varying expression of the eNOS protein in different areas of the aneurysmal aorta of BAV and TAV patients. While eNOS levels are indeed lower in the BAV proximal aorta than in its TAV counterpart, the BAV concavity features a higher eNOS expression than the TAV concavity, although this difference was not significant in our experiments ($P = 0.07$). The eNOS protein amount in the convexity and the distal aorta seems to be on equal levels in BAV and TAV.

The upregulation of eNOS mRNA expression by fluid shear stress is well established [7]. Recently Barker et al. quantified shear stress in BAV and control patients by phase

Table 3: Protein levels of 35 apoptosis-related proteins after treatment of HAECss with 1 mM L-NAME.

Protein	Control average	1 mM L-NAME average	Change	P
Bad	62.83 ± 4.88	66.76 ± 12.67	3.92	0.661
Bax	52.36 ± 16.36	54.62 ± 12.14	2.26	0.878
Bcl-2	35.77 ± 6.52	40.28 ± 4.95	4.51	0.373
Bcl-x	20.18 ± 5.18	17.03 ± 3.48	−3.16	0.410
Pro-Caspase 3	92.79 ± 10.67	89.73 ± 9.55	−3.06	0.756
Cleaved Caspase 3	29.29 ± 7.84	23.81 ± 3.32	−5.48	0.392
Catalase	58.39 ± 12.35	55.87 ± 6.19	−2.53	0.803
clAP-1	35.59 ± 9.16	27.73 ± 16.97	−7.87	0.560
clAP-2	15.76 ± 3.62	16.86 ± 5.57	1.10	0.813
Claspin	18.69 ± 2.87	18.00 ± 2.51	−0.69	0.745
Clusterin	35.62 ± 11.69	39.72 ± 4.57	4.10	0.660
Cytochrome c	62.37 ± 11.07	84.29 ± 6.92	21.92	0.069
TRAIL R1/DR4	56.01 ± 3.15	57.91 ± 12.80	1.91	0.833
TRAIL R2/DR5	88.48 ± 1.03	91.83 ± 8.18	3.35	0.516
FADD	72.14 ± 8.15	72.21 ± 7.40	0.07	0.989
Fas/TNFSF6	41.58 ± 7.37	34.54 ± 9.89	−7.04	0.303
HIF-1a	28.35 ± 7.46	26.07 ± 6.38	−2.29	0.735
HO-1/HMOX1/HSP32	30.43 ± 6.38	26.90 ± 12.93	−3.53	0.728
HO-2/HMOX2	35.86 ± 1.94	34.72 ± 11.64	−1.15	0.887
HSP27	41.67 ± 9.25	44.93 ± 11.15	3.26	0.699
HSP60	62.68 ± 8.65	70.54 ± 22.29	7.87	0.640
HSP70	65.35 ± 7.42	69.88 ± 17.35	4.53	0.727
HTRA2/Omi	78.98 ± 5.64	84.13 ± 9.00	5.15	0.482
Livin	12.39 ± 2.85	17.58 ± 6.45	5.18	0.315
PON2	41.23 ± 2.47	44.50 ± 12.63	3.27	0.711
p21/CIP1/CDNK1A	30.05 ± 11.24	35.52 ± 9.31	5.47	0.611
p27/Kip1	30.42 ± 3.67	29.70 ± 4.48	−0.73	0.777
Phospho-p53 (S15)	22.92 ± 6.58	25.48 ± 6.03	2.56	0.680
Phospho-p53 (S46)	8.84 ± 4.14	9.08 ± 2.81	0.24	0.948
Phospho-p53 (S392)	4.06 ± 2.69	5.01 ± 6.63	0.95	0.844
Phospho-Rad17 (S635)	12.24 ± 3.83	13.16 ± 9.86	0.92	0.900
SMAC/Diablo	63.31 ± 14.25	63.90 ± 12.04	0.59	0.956
Survivin	23.28 ± 1.48	21.28 ± 3.96	−2.00	0.443
TNF RI/TNFRSF1A	27.19 ± 3.24	25.24 ± 4.74	−1.95	0.599
XIAP	53.37 ± 7.73	51.67 ± 3.93	−1.70	0.769

All values are in percent of the calibrator (positive control). The change is given in percent points of the calibrator. The data were acquired in three independent experiments.

contrast MRI and thereby revealed a significant difference in aortic shear stress distribution between the groups [10]. Moreover, they found the hemodynamic forces to be significantly higher in the concavity of BAV patients than in control patients, while wall shear stress in the convexity was equal in both groups. These findings suggest an upregulation of eNOS in the BAV concavity by fluid shear stress which coincidences with our data. It is reasonable to assume that the altered valve morphology in BAV results in a different distribution of aortic wall shear stress and therefore leads to a locally different eNOS expression compared to TAV.

Apoptosis of vascular smooth muscle cells (VSMCs) seems to account for the formation of aneurysm in BAV [2, 3, 22]. In a recent study we provided evidence that VSMCs show a different behaviour in respect to apoptosis in the concave versus the convex sites in BAV ascending aortic aneurysm [11]. We found that the inhibition of caspase 3 leads to an increased protection against apoptosis in the BAV concavity compared with the convexity. The detection of a significantly higher eNOS protein expression in the BAV concavity than in the BAV convexity appears to be consistent with these earlier results, since nitric oxide generated by eNOS is known to inhibit apoptosis by s-nitrosylation of caspases [13]. Therefore it seems that an elevated eNOS expression in the BAV concavity can contribute to an increased protection against VSMC apoptosis in this aortic area.

To analyze the effect a dysregulation of eNOS could have on VSMC and HAEC apoptosis, we treated VSMCs which

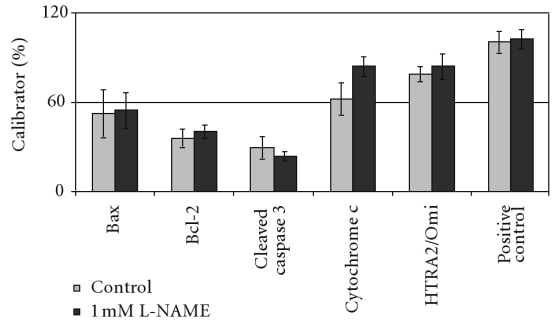

FIGURE 3: Change in concentration of apoptosis-related proteins after treatment with L-NAME. Bars display the change in concentration of Bax, Bcl-2, cleaved caspase 3, cytochrome c, HTRA2/Omi, and the positive control after treatment of HAECs with 1 mM L-NAME for 1 h.

originated from aortic tissue, as well as primary HAECs with 1 mM of the NO synthase inhibitor L-NAME. Of note, L-NAME does not specifically inhibit eNOS but all NO synthases. Although eNOS is the primary source of NO in the aorta, other NO synthases are known to be active in VSMC under certain conditions [23]. Therefore the effect of L-NAME treatment cannot solely be attributed to eNOS inhibition. We are also aware that eNOS is mainly expressed in endothelial cells. However, it was shown that VSMCs do express eNOS and that the produced NO is physiologically relevant [24, 25]. Inhibition of NO synthesis in VSMC resulted in an increase in cytosolic HTRA2/Omi proteins. HTRA2/Omi is a mitochondrial serine protease, which initiates apoptosis if released into the cytosol [26]. Treatment of HAECs with L-NAME did not account for any changes in cytosolic HTRA2/Omi levels but the cytosolic amount of cytochrome c increased after NO synthase inhibition although the difference was not significant ($P = 0.069$). The increased presence of mitochondrial proteins in the cytosol after L-NAME treatment indicates an inhibitory effect of eNOS produced NO on the intrinsic apoptosis pathway. This pathway, opposed to the death receptor triggered extrinsic pathway, is activated by proapoptotic Bcl-2 proteins, which cause mitochondrial outer-membrane permeabilization and thus cause a release of mitochondrial cytochrome c into the cytosol. Here cytochrome c facilitates assembly of the apoptosome, which activates procaspase-9 and therefore initiates caspase cascades [26, 27]. We detected neither in VSMCs nor in HAECs an increase in the Bax/Bcl-2 ratio after treatment with L-NAME. This is astonishing, since an increase in the ratio of Bax to Bcl-2 normally indicates an activation of the intrinsic apoptosis pathway [28].

NO is reported to have pro- and antiapoptotic effects depending on its concentration, at which physiological concentrations act antiapoptotic [29, 30]. A study by Liu et al. showed that excessive amounts of NO can lead to an increase in HTRA2/Omi release from mitochondria and endothelial cell apoptosis by formation of peroxynitrite [31]. Here we show that a reduction of physiological NO concentration by inhibition of NO synthesis can result in cytosolic accumulation of HTRA2/Omi. This presumably leads to apoptosis of VSMCs. The locally different eNOS expression we found in BAV patients can account for a lower NO concentration in certain aortic areas and therefore lead to upregulation of HTRA2/Omi which might initiate apoptosis in VSMCs.

We are aware that our sample number, especially for TAV patients, is limited and our data display a high standard deviation. However, Barker et al. also discovered a high standard deviation when they determined aortic wall shear stress in BAV patients [10]. Hence it could be hypothesized that a high standard deviation in eNOS expression is due to a high standard deviation of aortic wall shear stress which influences expression of eNOS. Fluctuation in shear stress between BAV patients might be due to the morphology of the aortic valve, as BAV is known to feature heterogeneous phenotypes depending on the morphological type of BAV [32–34].

In summary, we showed a locally variable eNOS protein expression in BAV aortic aneurysms than in TAV aortic aneurysms. In addition, we showed a cytosolic accumulation of the proapoptotic serine protease HTRA2/Omi after treatment of VSMCs with L-NAME. Taken together, these results indicate a locally different eNOS protein expression, which is probably caused by variations in aortic wall shear stress between BAV and TAV aortic aneurysm patients, which in turn might be caused by differences in BAV and TAV valve morphology. We conclude that eNOS dysregulation can lead to a dysregulation of NO in certain areas of the aorta, which can lead to VSMC apoptosis mediated by HTRA2/Omi. Thus the low eNOS protein expression in the BAV proximal aorta may confer susceptibility to aortic aneurysm formation.

Authors' Contribution

Both authors have contributed equally to this paper.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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3.3 Publication III: MicroRNA 208 in atrial fibrillation

MicroRNAs (miRNA) are small non-coding RNA of approximately 22 nucleotides length. They are able to regulate the translation of their target mRNA, either by a block of translation or by the induction of mRNA degradation[118, 119]. MiRNA mediated control of translation seems to play an important role in the pathogenesis of atrial fibrillation[93, 120, 121]. The cardiac specific miRNA 208a has been previously associated with cardiac rhythm disorders in mice. Interestingly not only transgenic overexpression but also knock down of miRNA 208a can cause conduction delay and arrhythmias in these animals[96]. The miRNA is located intronic to the *Myh6* gene, which encodes for the fast myosin heavy chain. MiRNA 208a is part of a miRNA family with two other members, intronic within the slow myosin genes[122, 123]. MiRNA 208a regulates muscle growth and hypertrophy by targeting the mRNAs of *thyroid hormone–associated protein 1* and *myostatin* and is also associated with cardiac fibrosis, which is regularly seen during the course of atrial fibrillation[96, 122, 124]. Additionally it is required for the proper expression of Connexin 40, a cardiac gap junction protein linked to the pathogenesis of atrial fibrillation in animal models[96, 125, 126].

These data suggest a role for miRNA 208a in human atrial fibrillation and therefore its expression was quantified in left atrial appendage tissue of 19 patients. The patient collective consisted of 2 patients with paroxysmal atrial fibrillation, 10 patients with persistent atrial fibrillation and 7 patients with long-standing persistent arrhythmia.

The relative miRNA 208a expression was on a medium level in paroxysmal atrial fibrillation, high in patients with persistent and low in patients with long-standing persistent arrhythmia. The difference between persistent and long-standing persistent atrial fibrillation was significant with $p = 0.02$. These results suggest a change of miRNA 208a expression over the course of the arrhythmia, with a decline of the miRNA from persistent to long-standing persistent atrial fibrillation.

-III-

MicroRNA 208 in atrial fibrillation

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Own contribution:

- Part of the study design
- Isolation of RNA from atrial tissue
- cDNA synthesis
- relative quantification of miRNA 208a
- Statistical analysis
- Co-writing of the manuscript

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Research Article Open Access

MicroRNA 208 in Atrial Fibrillation

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Abstract

MicroRNAs (miRNAs) are critical regulators of most major cellular processes and seem to play a vital role in the pathogenesis of numerous diseases including atrial fibrillation, the most commonly encountered cardiac rhythm disorder. Among the several miRNAs that appear to be involved in pathogenesis of atrial fibrillation, miRNA 208a is linked to fibrosis and proper cardiac conduction.

We quantified the expression levels of miRNA 208a in left atrial appendage tissue of patients with paroxysmal (n=2), persistent (n=10), and long-standing persistent (n=7) arrhythmia using quantitative PCR. In paroxysmal atrial fibrillation, miRNA 208a was expressed moderately, whereas the expression was enhanced in persistent atrial fibrillation and significantly reduced in long-standing persistent atrial fibrillation. The difference between persistent and long-standing persistent atrial fibrillation was significant at p=0.02.

The findings from our study suggest a decline in miRNA 208a expression with ongoing arrhythmia, possibly preceded by a rise in expression from paroxysmal to persistent atrial fibrillation or even long-standing persistent. The significant changes in miRNA 208a expression over the course of the disease may be used as an additional diagnostic tool to monitor the progression of atrial fibrillation.

Keywords: MicroRNAs; Atrial fibrillation; Arrhythmia; Expression

Introduction

Atrial fibrillation is the most common form of cardiac atrial arrhythmia, with a prevalence of 0.4%–1% in the general population and 5.5% in patients over 55 years [1-3]. According to Go et al., the estimated prevalence will become more than double by the end of 2050. Atrial fibrillation increases the risk of stroke and is responsible for more than half of the deaths resulting from cardiovascular diseases [2]. The arrhythmia also accounts for stroke and stroke related death and generally doubles the mortality from cardiovascular disease [4,5]. Different pathological mechanisms are believed to be associated with the development and maintenance of atrial fibrillation. Several factors have been implicated in the pathogenesis of atrial fibrillation such as atrial electrical remodeling via changes in gene expression associated with gap junction proteins (connexins), structural remodeling caused by fibrosis of the myocardium, and inflammation [6,7]. Recent studies have highlighted the role of microRNAs (miRNAs) in the pathogenesis of atrial fibrillation [8]. Several miRNAs have contributed to the pathogenesis of atrial fibrillation by regulating the availability of their target mRNAs and the corresponding proteins. For example, the overexpression of muscle-specific miRNA 1 slows down cardiac conduction by inhibiting the translation of the calcium channel subunit Kir2.1 and connexin 43 [9].

Members of the miRNA 208 family (miRNA 208a and miRNA 208b) are considered to be heart specific. miRNA 208a, which is located intronic to the Myh6 gene, is associated with cardiac conduction in mice as well as cardiac hypertrophy and fibrosis in both humans and mice [10,11]. The overexpression of miRNA 208a in transgenic mice was sufficient to induce cardiac arrhythmia, whereas mice lacking miRNA 208a exhibited abnormal cardiac conduction [11]. There is also evidence that miRNA 208 plays a role in cardioprotection by post-conditioning after ischemia/reperfusion injury as well as in differentiation of cardiac embryonic stem cells, demonstrating it various roles in cardiac biology [12,13].

We therefore hypothesized that dysregulation of this miRNA plays a crucial role in inducing atrial fibrillation. Therefore, in this study we quantified the expression of miRNA 208a in the left atrial appendage tissue of patients suffering from atrial fibrillation as well as commercial heart tissue RNA obtained from healthy donors.

Material and Methods

Sample collection

All experiments performed with human tissue strictly adhered to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the institutional ethics committee, and written informed consent was obtained from each patient. Specimens obtained during surgery were frozen immediately. A total of 19 samples of left atrial appendage tissue were obtained from patients suffering from atrial fibrillation. In addition, 3 samples of aortic tissue showing no signs of atrial fibrillation were collected. We compared

normal left atrial adult total RNA (purchased from US Biological, Massachusetts, USA) with 3 of our samples by employing miRXplore Microarrays (Miltenyi Biotech, Bergisch Gladbach, Germany). According the manufacture's recommendations and as described previously [14]. To validate the microarrays data set in all 19 samples we picked miRNA208, the most differentially expressed one.

RNA isolation

Isolation of total RNA was achieved using the Trizol reagent (Life Technologies) according to the manufacturer's instructions. In brief, 100 mg of tissue was homogenized with 1 mL of Trizol solution using a tissue lyser (Qiagen, Hilden, Germany). Then 0.2 mL of chloroform was added to the homogenized solution and the lysate was centrifuged to accomplish phase separation. The aqueous phase containing the total RNA was carefully removed and subsequently precipitated by adding isopropyl alcohol, washed, and re-suspended in nuclease-free water.

cDNA synthesis

For synthesis of cDNA, the TaqMan MicroRNA Reverse Transcription Kit was utilized together with the reverse transcription primers from the human miRNA 208 assay (Assay ID 000511; Applied Biosystems, Weiterstadt, Germany). Reverse transcription was performed following the manufacturer's protocol with 10 ng of RNA as the input amount.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Relative quantification of miRNA 208a expression was performed on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) with nuclear RNA RNU48 as the internal control (Assay ID 001006; Applied Biosystems). The samples were run in triplicates with 1.3 µL of the corresponding cDNA in a total volume of 20 µL. Control without reverse transcriptase was run for each sample and control without template was run for every cDNA synthesis and PCR reaction. The thermal cycle profile consisted of one initial 2 min step at 50°C, one 10 min denaturation step at 95°C, and 40 cycles of 15-s denaturation at 95°C and 60s elongation at 60°C.

Statistical analysis

Statistical analysis was performed using excel/WIN STAT and SPSS. The Data obtained from each experiment are presented as mean ± standard deviation. Differences between miRNA expression levels and other continuous variables were tested with two-sided Mann–Whitney U-test and differences in gender distribution were analysed using Fisher's exact-test. For analysing the association among different parameters, Pearson's correlation coefficient was utilized. Significant differences were considered at $p<0.05$.

Results

Patients characteristics

Patients diagnosed with atrial fibrillation were divided into sub-categories according to the latest guidelines established by the European Society for Cardiology in 2012. Atrial fibrillation episodes shorter than 48 h were considered paroxysmal, whereas arrhythmias lasting for more than 7 days or being treated pharmacologically or electrically within these 7 days were considered persistent. Atrial fibrillation persisting for more than 1 year was classified as long-standing persistent. Patient characteristics are shown in (Table 1).

Patient Characteristics	Atrial fibrillation (AF)-Classifications		
AF	paroxysmal (n=2)	persistent (n=10)	long persistent (n=7)
Age, ys	73.50 ± 0.707	65.2 ± 4.2	66.3 ± 3.6
Gender, male: female	1:01	8:02	4:03
BMI	27.17 ± 0.00	31.27 ± 1.96	29.87 ± 2.28
LA-D, mm	45	47.43 ± 3.43	51.86 ± 2.09
LV-EF, %	60	52.20 ± 4.63	53.29 ± 5.88
NYHA	3.0 ± 0	3.10 ± 0.18	3.00 ± 0.218
CAD	0	5 (50%)	1 (14.3)
AoV-Vitium	1 (50%)	2 (20%)	1 (14.3)
TV-Vitium	1 (50%)	3 (30%)	4 (57.1)
MV-Vitium	2 (100%)	10 (100%)	7 (100%)
VD	1 (50%)	4 (40%)	6 (85.7%)#
CHF	2 (100%)	10 (100%)	6 (100%)
aHTN	1 (50%)	10 (100%)	5 (71.4%)
DM	1 (50%)	2 (20%)	1 (14.3%)
Stroke	1 (50%)	2 (20%)	5 (71.4%)*
CHA2DS2-VASc	6	4.1 ± 0.53	5.00 ± 0.72
CHADS2	2	2.7 ± 0.26	2.29 ± 0.42

Table 1: Patients' characteristics, *$p<0.05$, #$p\approx 0.05$

AF: Atrial Fibrillation; BMI: Body Mass Index; LA-D mm: Left Atrium Diameter in Millimetre; LV-EF%: Left Ventricle Ejection Fraction in Percentage; NYHA: New York Heart Association; CAD: Coronary Arterial Disease; Aov-Vitium: Aortic Valve Vitium; TV-Vitium: Tricuspid Valve Vitium; MV-Vitium: Mitral Valve Vitium; VD: Vascular Disease; CHF: Chronic Heart Failure; Ahtn: Arterial Hypertension; DM: Diabetes Mellitus; CHA2DS2-Vasc: Congestic Heart Failure/ Insufficiency

Micro RNA 208a expression

The relative expression level of miRNA 208a was 2.33 ± 1.99-fold of calibrator in atrial fibrillation tissue, whereas it was 1.4 E−4 ± 2.4 E−4 in RNA obtained from healthy donors control aortic tissue RNA (data not shown), thus confirming the cardiac specific expression of this miRNA.

The comparison among duration-based atrial fibrillation sub-categories revealed miRNA 208a expression levels of 2.32 ± 2.12 in paroxysmal patients, 3.26 ± 2.30 in persistent atrial fibrillation, and 1.14 ± 0.67 in long-standing persistent arrhythmias (Figure 1). The difference in miRNA 208a expression levels between persistent and

long-standing persistent atrial fibrillation proved to be significant with p=0.02. All other differences were not significant.

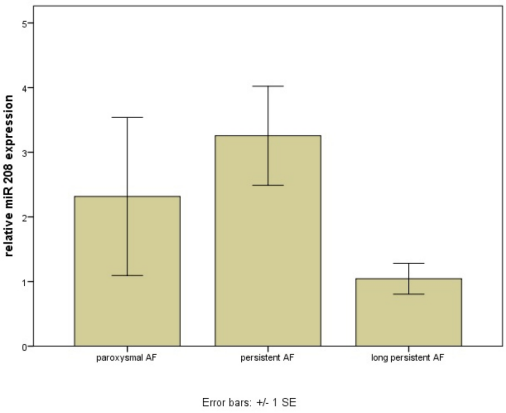

Figure 1: Relative miRNA 208a expression in atrial fibrillation categories. The average relative expression of miRNA 208a in paroxysmal, persistent, long-standing persistent atrial fibrillation is shown. The error bars represent standard deviation, and the asterisk indicates a significant difference between the average miRNA 208a expression in persistent and long-standing persistent atrial fibrillation. Differences in age, sex, or BMI between the groups were not significant; comparison between one group and all other groups treated as a whole revealed no significant differences as well. There was also no significant correlation between age or BMI and miRNA 208a expression.

Discussion

The results of our study demonstrate a significantly higher miRNA 208a expression in patients with a history of persistent atrial fibrillation tissue than in patients with long-standing persistent atrial fibrillation tissue. Additionally our data suggest a medium expression level of miRNA 208a in paroxysmal atrial fibrillation and a very low expression in aortic tissue (data not shown). These results indicate a decline in miRNA 208a expression over time from persistent to long-standing persistent atrial fibrillation.

There is growing evidence indicating changes in gene expression associated with sustained atrial fibrillation. Chung et al. found higher levels of C-reactive protein (CRP), an inflammatory marker, in persistent arrhythmia than in paroxysmal arrhythmia, indicating a stepwise higher inflammatory response in more active atrial fibrillation [15].

miRNA 208a is part of the miRNA network and the miRNAs from this network are encoded intronic and coexpressed with myosin heavy chain genes. The Myh6 gene encodes the fast α-myosin heavy chain (αMHC) and also co-expresses miRNA 208a, whereas the slow myosin genes Myh7 and Myh7b encode the microRNAs 208b and 499, respectively [10,11]. In mice, where αMHC is the predominant isoform, miRNA 208a is the dominant miRNA and is utilized for the expression of the slow myosin isoforms. In humans, αMHC is predominantly expressed in the cardiac atrium, whereas the slow isoform β-myosin heavy chain (encoded by the Myh7 gene) is the main form in the ventricle [10,16]. It remains unclear whether miRNA 208a fulfills a similar role related to the expression of slow myosin isoforms in humans as in mice. In a study related to contractile dysfunction in permanent atrial fibrillation, Mihm et al. reported decreased expression of αMHC in human atria, whereas βMHC expression was enhanced [17]. This switch in the αMHC/βMHC ratio is observed in rodent models of cardiac dysfunction and also in human heart failure leading to reduced contractile velocity [18-20]. The decline in miRNA 208a expression that we observed in case of enduring atrial fibrillation may be partly attributed to the switch in the ratio between fast and slow myosin heavy chain isoforms. However, if miRNA 208a performs analogous role in humans as in mice, there should be an upregulation of miRNA 208a expression prior to the increase in slow myosin heavy chain expression. There may be an upregulation of miRNA 208a at the beginning of cardiac arrhythmia, as indicated by the rise in miRNA 208a expression levels from paroxysmal to persistent atrial fibrillation in our study, although this difference was not significant. A comparison between atrial miRNA 208a expression in patients with sinus rhythm and different types of atrial fibrillation may provide useful information regarding miRNA expression during the course of atrial fibrillation.

Callis and colleagues reported that the transgenic overexpression of miRNA 208a as well as the lack of miRNA 208a expression in mice is sufficient to induce arrhythmias [11]. Therefore the balance of miRNA 208a expression seems to play a pivotal role for proper cardiac conduction in mice. Our results suggest the possible rise and decline of miRNA 208a expression levels with ongoing atrial fibrillation and hence show that imbalances in miRNA 208a expression are associated with arrhythmias in humans as well.

Recently, circulating miRNA 208a has gained importance as a biomarker for detecting cardiovascular diseases [21]. Wang et al. demonstrated that circulating miRNA 208a can be used to detect acute myocardial infarction with high sensitivity and specificity [22]. In addition, overexpression of miRNA 208a predicts poor clinical outcome in patients with dilated cardiomyopathy [23]. Therefore, our results suggest a biomarker role for circulating miRNA 208a to support the diagnosis of the severity of atrial fibrillation status, keeping in mind, that a significant proportion of patients gets misclassified by clinical categorization [24].

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4. Discussion

4.1 HMGA2 overexpression in the dissecting aorta

A significant upregulation of the *HMGA2* gene was demonstrated in aortic tissue of acute aortic dissection cases as a part of this study. Other cases of cardiovascular disease and heart valve control samples featured a much lower expression of the gene, albeit some thoracic aortic aneurysm cases displayed an increased *HMGA2* expression. These results indicate an association between *HMGA2* upregulation and acute aortic dissection, as the gene is normally not expressed in the healthy aorta of human adults[127]. Additional experiments proved the presence of the HMGA2 protein, as well as the EMT markers SNAI1 and Vimentin in the endothelium of the *vasa vasorum* but also in the *tunica intima*, thereby suggesting the transition of these endothelial cells to mesenchymal cells. The ability of HMGA2 to elicit EMT is already known from cancer biology. Here *HMGA2* is re-expressed as a reaction to TGF-β induced Smad-signaling and then facilitates the expression of EMT promoting factors like SNAI1, Slug and Twist[33, 105, 106]. The same process seems to occur in acute aortic dissection, particularly since a dysregulation of the TGF-β pathway is seen in many forms of aortic dissection[16]. It is noteworthy that it is not really an EMT taking place in the aorta, since the cells involved are not epithelial cells, but an Endothelial to Mesenchymal Transition (EndMT). This form of cell differentiation is generally assumed to be similar to EMT, including activation by TGF-β signalling and upregulation of SNAI1, and is also involved in cancer progression and fibrosis[128-130].

Two possible conclusions can be drawn from the *HMGA2* upregulation and concomitant EndMT in acute aortic dissection tissue. These events either contribute to the pathogenesis of the disease or are a direct result of the injury. In the first case the EndMT elicited by HMGA2 would cause a weakening of the endothelial layer through the loss of endothelial cells. This would facilitate the tearing of the aortic layers during dissection. The HMGA2, SNAI1 and Vimentin proteins were mainly detected in the *vasa vasorum* endothelium and indeed tearing of these vessels can cause intramural hematoma, which are considered to be a precursor of aortic dissection[131]. In this case haemorrhage in the aortic *tunica media* can lead to the dissection without the presence of an intimal tear. Intramural hematoma constitute 10

to 30% of all acute aortic dissection cases and feature a higher rate of aortic rupture than normal dissections due to their location closer to the *tunica adventitia*[132, 133].
Additional linkage between aortic disease and *HMGA2* was discovered in a genome wide association study by Vasan and colleagues[134]. A SNP near the gene was strongly linked to the aortic diameter, which is in accordance with the association of *HMGA2* with growth in mice and man[135-139]. Therefore HMGA2 overexpression in the aortic endothelium might lead to an enlargement of the aortic diameter, which increases the risk for aortic dissection[17]. This could represent a further aspect in the relationship between *HMGA2* expression and aortic dissection. As an interesting side note *HMGA2* expression is not only induced by TGF-β signalling, but HMGA2 can also enhance TGF-β signalling by increasing expression of the *TGF-β type II receptor* (*TGFβRII*), which could lead to circular proliferation of the signal[140].
There are of course some considerations that render an involvement of the *HMGA2* induced EndMT in the pathogenesis of aortic dissection unlikely. While not all aortic dissection cases featured a vastly increased *HMGA2* expression, the frequency was certainly above 10 to 30 %. Therefore the presence of intramural hematoma can not be the reason for dissection in the majority of cases, especially since patients with intramural hematoma are reported to be in their mid-seventies on average, while the patients in this study featured a mean age of 59.6 years[141]. Of course HMGA2 and the EMT markers were also detected in the *tunica intima*, a factor that could lead to a standard type A dissection by weakening the aortic endothelium. Another aspect that makes the participation of HMGA2 upregulation in aortic dissection pathogenesis seem dubious, is the fact that aortic dissections are considered to evolve from aortic aneurysms. One would expect an increased *HMGA2* expression in these patients, contributing to the weakening of the aortic wall and finally leading to the dissection. Actually aortic aneurysm cases in this study did feature a slightly higher *HMGA2* expression than other non-dissecting tissues, although the difference was not significant. There was some overlap between the aneurysm cases with the highest *HMGA2* expression and the dissection cases with a lower expression but the average expression in aortic dissection was over 20 times higher than in aortic aneurysms.
In summary there are some facets, which let this pathomechanism seem questionable and therefore favour the other explanation for the *HMGA2* upregulation:

A reaction to the injury from the dissection. Indeed there is a form of EMT associated with wound repair and tissue regeneration, labelled as type 2 EMT, during which fibroblasts are generated in order to reconstruct the tissue[142]. In keratinocytes the process of wound re-epithelialisation is triggered by the transcription factor Slug, a mediator of EMT[143]. Even *HMGA2* expression is linked to wound recovery: Chin and colleagues found a dramatic increase in *HMGA2* mRNA after balloon injury of rat carotid arteries, while Monzen et al. detected *HMGA2* re-expression in the mouse myocardium after myocardial infarction[144, 145]. It therefore seems likely that the increase in *HMGA2* expression in acute aortic dissection and the accompanying EndMT can be attributed to the tearing of the aorta and the following tissue regeneration.

As a further result of this study the *let-7d* expression of 17 acute aortic dissection tissue samples was quantified and compared to the *HMGA2* expression in the same samples. There was no significant correlation between the two data sets, indicating that *let-7d* does not regulate *HMGA2* in these cases of aortic dissection, otherwise one would expect a negative correlation. The miRNAs of the let-7 family are known to repress *HMGA2* expression, a mechanism which is obliterated in some cancers[146, 147]. In hepatocellular carcinoma cell lines *let-7g* regulates EMT by repression of *HMGA2* and *K-Ras*[148]. Another let-7 variant might be responsible for the repression of *HMGA2* in aortic dissection too or alternatively the missing regulation might be the reason for the *HMGA2* upregulation.

The average *HMGA2* expression in this study was thrice as high in women with acute aortic dissection as in men with the disease, which might be explained by the fact that the women were also significantly older. This coincides with a report of women being significantly older when suffering from aortic dissection than men[149]. The women also displayed significantly higher in-hospital mortality than men and an altered spectrum of symptoms.

Further studies about the upregulation of *HMGA2* and the EndMT in aortic dissection should focus on the question whether these events are cause or consequence of the disease. A suitable approach to verify this problem would be the generation of a transgenic mouse model. A mouse overexpressing *HMGA2* in the vascular endothelium could be generated by coupling *HMGA2* with the *Tie-2* promotor, which causes endothelium-specific gene expression as demonstrated in various mice[150, 151].

The mice generated this way should develop acute aortic dissections if HMGA2 is part of the aortic dissection pathogenesis.

Additionally the *HMGA2* expression in whole blood samples from acute aortic dissection cases and control samples should be quantified. The possibility to detect *HMGA2* mRNA in blood samples has already been demonstrated in the peripheral blood of breast cancer patients[152]. This could be important since a reliable biomarker for acute aortic dissection is still lacking[153]. Aortic dissection is rare and sometimes accompanied by atypical symptoms, which results in a delay of diagnosis of the life-threatening disease[154]. To aid the diagnosis a variety of blood based biomarkers have been proposed, such as D-dimers, soluble Elastin fragments, serum smooth muscle Myosin heavy chain or Calponin but none of them is completely reliable[155-159]. The increased *HMGA2* expression in aortic dissection suggests a possible role as a biomarker for the mRNA, which could be part of a blood based biomarker profile comprising several biomarkers for an increased sensitivity and specificity.

4.2 Spatial differences in eNOS protein levels between BAV and TAV aortas

The question about the pathological mechanism leading to the development of aortic aneurysm in BAV has provoked a lot of controversy. Two main theories are discussed: One assumes a common congenital defect, which is responsible for the development of the BAV and the weakening of the aortic wall; the other deems hemodynamic changes through the altered valve morphology to cause shear stress and therefore weaken the aortic wall. The difference is not only of scientific importance but has also influence on the health of many patients undergoing surgery for BAV related aneurysm, as these develop either from a diseased aortic wall or from the malformed aortic valve and therefore the correct surgical treatment is still subject of discussion[58, 160].

The first theory sees the same developmental defect leading to the development of BAV and the weakening of the aortic wall. This assumption seems reasonable, since the aortic valve and the ascending aorta share a common neuroectodermal origin and therefore a genetic defect could apply to both tissues[161, 162]. Additionally cystic

medial necrosis, characterised by elastic lamina fragmentation, loss of smooth muscle cells and accumulation of mucoid material, is found not only in BAV associated aneurysms but also in many forms of heritable aneurysm disease, suggesting a common defect[115, 163]. Furthermore different BAV types seem to stem from different genotypes and therefore represent different forms of the disease[40]. A different genetic background of the BAV types could explain the fact that the L-R type is associated with more severe aortic root dilatation at a younger age[39]. There are two other arguments which seem to disprove the hemodynamic theory and therefore strengthen the genetic theory. The first one focuses on the assumption that only a diseased (stenotic or insufficient) BAV would lead to significant changes in hemodynamic and therefore the observation of Nistri et al., who found 52 % of young men with clinically normal BAV to have enlarged aortic dimensions, contradicts the hemodynamic theory[43]. The second argument is found in a study by Yasuda and colleagues, in which they demonstrated an ongoing increase in aortic diameter in a cohort of 13 BAV patients undergoing aortic valve replacement, which was comparable to BAV patients without aortic valve replacement[164]. These data indicate a progression of aortic dilatation even after the aortic valve, which would cause the altered hemodynamic, was replaced and consequently speak for the genetic nature of BAV aortopathy.

The second theory about the pathogenesis of aortic aneurysms related to BAV holds changes in hemodynamic caused by the deformed aortic valve for responsible. These alterations would cause abnormal shear stress on some areas of the aortic wall and thereby weaken the aorta. This theory is supported by several recent studies, which confirmed an abnormal blood flow pattern and an asymmetric distribution of shear stress in patients with BAV either by the use of magnetic resonance imaging or by computational analysis[165-169]. Moreover different BAV types induced different flow patterns, which might also explain the association of the L-R BAV type to more severe aortic dilatation[39, 168]. Further evidence comes from studies analysing apoptosis and extracellular matrix remodelling in different areas of the aorta. Della Corte and colleagues demonstrated asymmetric changes in medial necrosis, extracellular matrix protein expression and smooth muscle cell apoptosis in BAV aneurysm compared to controls, with the convexity of the aorta more severe affected in most cases[61, 62]. Mohamed et al. confirmed that inhibition of Caspase 3

protects vascular smooth muscle cells significantly more from apoptosis if they are derived from the BAV concavity than from the BAV convexity[170]. The same group detected asymmetric protein expression of matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinase (TIMPs), two proteins involved in extracellular matrix remodelling[171]. Taken together these studies indicate an abnormal hemodynamic in BAV, which may result in smooth muscle cell apoptosis and extracellular matrix remodelling, which in turn seems to be more severe at the convexity of the aorta than at the concavity. Additionally the two arguments outlined above, which seemingly disproved the hemodynamic theory, have been challenged by recent literature. Two studies seem to prove that the clinically normal BAV does produce an abnormal hemodynamic, which nullifies the argument that a high prevalence of aneurysms in patients with normal BAV supports the genetic theory[168, 172]. The progression in aortic dilatation after aortic valve replacement demonstrated by Yasuda and colleagues has been questioned by a recent study of Charitos et al., who did not detect a significant increase in aortic diameter after aortic valve replacement in a cohort of 360 BAV patients compared to the general population[173].

Many of these recent observations seem to support the hemodynamic theory better than the genetic theory, although the extensive amount of extracellular matrix remodelling and gene expression changes found at certain aortic sites in BAV seems to suggest further mechanisms besides simple shear stress to be involved.

As a part of this study the expression of the eNOS protein was quantified at four different sites of the aorta in BAV and TAV patients suffering from aortic aneurysms. The spatial distribution of the eNOS protein within the aorta proved to be different between BAV and TAV patients, with the eNOS amount being higher in the BAV concavity and significantly lower in the BAV proximal aorta than in its TAV counterpart. The eNOS protein expression was significantly higher in the concavity than in the proximal aneurysm and the convexity of BAV patients, while the differences in TAV aortic areas where not significant. These results suggest a spatial dysregulation of eNOS expression in BAV when compared to TAV.

The eNOS is an enzyme which produces nitric oxide (NO), a messenger molecule of the vascular system with manifold influence on vascular homeostasis. The NO produced by eNOS causes relaxation of the vessel wall through activation of the soluble guanylate cyclase[174, 175]. The NO molecule is also able to regulate gene

expression and the translation of mRNA and therefore influence cellular activity[176-178]. Furthermore expression and activity of the eNOS are known to be upregulated by the influence of fluid shear stress on endothelial cells[179, 180].

Therefore the spatial differences in eNOS protein expression between BAV and TAV suggest a role for the enzyme in shear stress induced BAV aortopathy. It seems probable that the asymmetric hemodynamic in BAV would lead to an irregular distribution of shear stress around the aorta. This would give rise to the spatial dysregulation of eNOS expression seen in the results of this study, which in turn would provoke an imbalance of NO distribution in the different aortic areas. The resulting changes in gene expression and mRNA translation could be sufficient to explain the asymmetric extracellular matrix remodelling and smooth muscle cell apoptosis observed in BAV-related aneurysms and therefore present an additional mechanism involved in the development of ascending aortic aneurysm in BAV disease.

The NO released to the vasculature can modify the apoptotic behaviour of the surrounding cells, as it directly inhibits caspases[113]. In an animal model mice deficient of eNOS displayed increased cardiomyocyte apoptosis and caspase 3 activity, suggesting an anti-apoptotic effect of the NO produced by eNOS[181]. In fact basal levels of NO seem to protect from apoptosis, while high NO concentration can act pro-apoptotic[182]. These properties of NO seem relevant, since the loss of vascular smooth muscle cells, detected during cystic medial necrosis in BAV aortopathy, stems from apoptosis[114]. Interestingly the BAV convexity, which is more severe affected by cystic medial necrosis according to della Corte et al.[61], displayed a significantly decreased eNOS expression compared to the concavity in the results of this study.

To further verify the effect of NO on aortic cell apoptosis, aortic endothelial cells and vascular smooth muscle cells, the two main cell types of the aorta, were subject to the inhibition of NO synthesis. Afterwards the expression of 35 apoptosis-related proteins was evaluated with a proteome profiler array. In vascular smooth muscle cells the cytosolic level of the serine protease HTRA2/Omi increased significantly after inhibition of NO synthesis, in aortic endothelial cells the cytosolic amount of Cytochrome c increased, albeit the difference was not significant with $p = 0.069$. Both proteins are released from the mitochondria as a consequence of the induction of the

intrinsic apoptosis pathway and elicit apoptosis once being present in the cytoplasm[116, 117]. Therefore NO in the aorta seems to inhibit the intrinsic apoptosis pathway, a mechanism which could be disturbed by the spatial dysregulation of eNOS. Consequentially the differences in eNOS protein expression in certain areas of the BAV aorta could lead to the diminishment of apoptosis protection in these areas and an increase in cystic medial necrosis, which would facilitate aneurysm development.

The results of the latter part of this study are of limited value, because the level of NO inhibition is not correlated to the decrease in eNOS expression and therefore does not necessarily represent the *in vivo* condition. Continuative studies should try to mimic the interactions between hemodynamic, eNOS expression and anti-apoptotic influence of NO. Commercially available flow chambers can simulate shear stress on cells by creating circular flow of cell medium and could be used to expose aortic cells to different degrees of shear stress, like normal shear stress (as in TAV), increased shear stress or decreased shear stress. The expression of eNOS and the concomitant NO production should be quantified for the various degrees of shear stress. Afterwards apoptosis could be induced in these cells, with calcium chloride for instance, and the susceptibility of the cells to apoptosis as well the exact apoptotic pathways should be determined to verify the relation between asymmetric shear stress and aortic cell apoptosis.

4.3 Differences in microRNA 208a expression over the course of atrial fibrillation

The expression of the miRNA 208a was quantified in left atrial appendage tissue of patients with atrial fibrillation during this study. The data was grouped according to the duration of the arrhythmia and the miRNA 208a expression was compared between paroxysmal, persistent and long-standing persistent atrial fibrillation. The miRNA expression was lower in paroxysmal than in persistent atrial fibrillation but the difference was not significant. The expression in long-standing persistent arrhythmias was even lower than in paroxysmal atrial fibrillation and significantly lower than in persistent atrial fibrillation with a p value of 0.02, which indicates a decline in miRNA

208a expression over time from persistent to long-standing persistent atrial fibrillation.

The miRNA 208a is part of the miRNA 208 family, comprising also miRNA 208b and miRNA 499. Those three miRNAs are located intronic to the myosin heavy chain genes *Myh6*, *Myh7* and *Myh7b* and are coexpressed with them. The miRNA 208a is located within *Myh6* coding for the fast α myosin heavy chain (MHC), while miRNA 208b and miRNA 499 are encoded within *Myh7* and *Myh7b*, respectively, coding for slow β MHC isoforms[123]. In healthy human adult atrium the α MHC is the dominant myosin heavy chain form[183], whereas cardiovascular disease is often accompanied by a switch to β MHC. A decrease in fast α MHC is reported in human heart failure, leading to a decline of cardiac contractility[184, 185]. The shift from fast α MHC to slow β MHC is also reported from chronic or permanent atrial fibrillation and is assumed to contribute to the contractile dysfunction, which arises with longer lasting arrhythmias[186-188]. This loss of contractility seemingly facilitates the sustaining of the arrhythmia together with electrical and structural remodelling of the atria[189].

So the decline in miRNA 208a expression seems to be a by-product of the decrease in α MHC it is coexpressed with. Therefore the question remains whether the change in miRNA 208a expression does contribute to the sustaining of the arrhythmia by itself, or if the ongoing atrial fibrillation can be attributed solely to the switch in dominant MHC isoforms.

In a study by Callis and colleagues mice with transgenic overexpression of miRNA 208a, as well as mice lacking the micro RNA, displayed arrhythmias and failure in cardiac conduction, indicating a role for miRNA 208a in atrial fibrillation[96]. Interestingly the same study reported miRNA 208a to be required for the expression of the gap junction protein Connexin 40, which seems to be decreased and distributed more laterally in sustained human atrial fibrillation[190-192]. Thus a decline of miRNA 208a expression with the ongoing arrhythmia could lead to a loss of Connexin 40 in the atrium, thereby altering conduction and sustaining the atrial fibrillation.

One important question not resolved by the results of this study is whether there is a difference in miRNA 208a expression between patients with atrial fibrillation and healthy controls. The group around Slagsvold et al. just very recently addressed this question in a study about miRNA in atrial fibrillation. They used an array based

approach to quantify the expression of all human miRNAs presented in the miRBASE 16.0 build[193]. Remarkably miRNA 208a was the only miRNA significantly downregulated in left and right atrium of atrial fibrillation compared to patients in sinus rhythm, thereby highlighting its importance in atrial fibrillation. A simultaneously published study by the same group compared expression of miRNA between the left and the right atrium of atrial fibrillation patients. Again miRNA 208a was one of the few miRNA differently regulated between the atria, with a significantly higher expression in the left atrium[194]. The patient collective in both studies comprised almost equally paroxysmal and chronic atrial fibrillation, which makes it is feasible that the difference in miRNA 208a expression between atrial fibrillation and sinus rhythm controls was caused by the decline in the miRNA in patients with longer lasting arrhythmias.

Further research on the subject should try to establish miRNA 208a as a blood-based biomarker for atrial fibrillation and therefore determine the expression of the miRNA in blood or serum of patients and controls. Many miRNAs, including miRNA 208a, can be detected in blood where they are either bound to proteins or enveloped in small membraneous particles like exosomes[195, 196]. In a recent study miRNA 208a was confirmed as a highly sensitive and specific biomarker for acute myocardial infarction, emphasising its usefulness as a blood-based marker[197]. The results of this study suggest a decrease of miRNA 208a expression over the course of ongoing atrial fibrillation, thus the miRNA could not only be used to detect the arrhythmia but also to discriminate between different durations of atrial fibrillation.

5. Summary

The results of three studies about gene expression differences in cardiovascular disease are presented within this thesis.

The first topic covers the involvement of the *HMGA2* gene in cellular processes found in acute aortic dissection, a disease characterised by the splitting of the aortic layers leading to a high mortality. Many forms of aortic dissection disease have a dysregulation of the TGF-β pathway in common, a signalling pathway, which is linked to HMGA2 induced epithelial to mesenchymal transition (EMT) in cancer. Since it seems probable that such a relation also exists in acute aortic dissection, the expression of the *HMGA2* gene was quantified in 19 samples of ascending aortic dissection tissue and 32 samples of other cardiovascular disease. Indeed aortic tissue of acute aortic dissection samples revealed a significantly higher *HMGA2* expression than aortic tissue of the other cardiovascular disease samples. Further on the HMGA2 protein was detected together with several markers of EMT in the aortic endothelium surrounding the *vasa vasorum*. These results suggest the upregulation of HMGA2 in the endothelium of aortic dissection tissue, accompanied by the transition of these endothelial cells to mesenchymal cells, a variant of EMT termed endothelial to mesenchymal transition (EndMT). It is not clear if this mechanism contributes to the pathogenesis of acute aortic dissection by weakening of the aortic endothelium, or if the EndMT is a reaction to the injury.

The second study focuses on differences of the spatial distribution of the eNOS protein within the aorta of patients with bicuspid aortic valve (BAV). The BAV is a common congenital defect of only two aortic valve leaflets compared to the three leaflets of a normal tricuspid aortic valve (TAV). The disease is associated with aneurysms and dissections of the ascending aorta, possibly generated by irregular hemodynamic and aortic wall shear stress through the malformed aortic valve. The amount of the eNOS protein was quantified in four different aortic sites of 14 aortic aneurysm patients with BAV and 5 with TAV. The eNOS protein level differed significantly between aortic areas in BAV, while the differences in TAV where not significant. Additionally the BAV proximal aorta featured significantly less eNOS protein than the corresponding area in TAV. A spatial dysregulation of the eNOS expression in BAV is implied by these data, probably caused by changes in shear

stress, which is known to regulate eNOS gene expression and protein activity. The nitric oxide produced by eNOS has an anti-apoptotic effect on the surrounding tissue and therefore the amounts of 35 apoptosis-related proteins were quantified with a proteome profiler array after inhibition of nitric oxide synthesis in cultured aortic cells. The cytosolic level of the pro-apoptotic serine protease HTRA2/Omi proved to be significantly increased after inhibition of nitric oxide synthesis. Since this protein is normally released from the mitochondria into the cytosol during the intrinsic apoptosis pathway, the nitric oxide produced by eNOS seems to have an inhibitory effect on the intrinsic apoptosis pathway. The results of this study indicate the spatial dyregulation of the eNOS protein in BAV, probably due to an altered hemodynamic, and the associated irregular nitric oxide distribution. A loss of the anti-apoptotic properties of nitric oxide could give raise to aortic cell apoptosis in certain aortic areas, a mechanism known to contribute to the pathogenesis of aneurysm and dissections in BAV.

The miRNA 208a was quantified in tissue of atrial fibrillation patients in the third study. Atrial fibrillation is the most common form of cardiac arrhythmia and bears an increased risk of stroke. Ongoing atrial fibrillation can sustain itself by electrical and structural remodelling of the atria and therefore it is classified according to the duration of the arrhythmia. The cardiac specific miRNA 208a was quantified in left atrial appendage tissue of 2 patients with paroxysmal atrial fibrillation, 10 patients with persistent and 7 patients with long-standing persistent atrial fibrillation. The miRNA expression was significantly decreased in long-standing persistent atrial fibrillation compared to persistent atrial fibrillation, suggesting a decline over the course of the arrhythmia. The miRNA 208a is co-expressed with the *Myh* 6 gene, coding for the fast α myosin heavy chain. A switch from fast α myosin heavy chain to slow β myosin heavy chain is known in atrial fibrillation and the decline in miRNA 208a might be a by-product of this alteration. Alternatively the change in miRNA 208a expression might contribute to sustaining of atrial fibrillation, since studies with transgenic mice suggest a role for the miRNA in cardiac conduction and arrhythmogenesis.

6. Zusammenfassung

Die Ergebnisse von drei Studien über Genexpressionsunterschiede in kardiovaskulären Erkrankungen werden in dieser Doktorarbeit präsentiert.

Die erste Studie umfasst die Beteiligung des *HMGA2* Gens an zellulären Prozessen, die während einer akuten Dissektion der Aorta auftreten, einer Erkrankung, die durch einen Riss zwischen den Aortenschichten und einer damit verbundene hohe Sterblichkeitsrate charakterisiert wird. Viele Formen der akuten Aortendissektion haben eine Dysregulierung des TGF-β Signalweges gemeinsam, einen Signaltransduktionsweg, der mit der HMGA2 induzierten epithelialen-mesenchymalen Transition (EMT) in Krebserkrankungen in Verbindung steht. Da es möglich erscheint, dass eine solche Verbindung auch in akuten Aortendissektionen existiert, wurde die *HMGA2* Expression in 19 Gewebeproben von Dissektionen der aufsteigenden Aorta und 32 Gewebeproben anderer kardiovaskulärer Erkrankungen quantifiziert. Gewebeproben von akuten Aortendissektionen wiesen in der Tat eine signifikant höhere *HMGA2* Expression als die Aortengewebeproben von anderen kardiovaskulären Erkrankungen auf. Weiterhin wurde das HMGA2 Protein zusammen mit verschiedenen EMT-Markerproteinen in den die *Vasa vasorum* umgebenen Endothelzellen detektiert. Diese Ergebnisse deuten auf die vermehrte Expression des HMGA2 Proteins im Endothel von Aortendissektionsgewebe und die gleichzeitige Transition dieser Endothelzellen zu mesenchymalen Zelle hin, eine EMT Variante, die als endotheliale-mesenchymale Transition bezeichnet wird (EndMT). Es ist nicht klar, ob dieser Mechanismus durch die Schwächung des Aortenendothels zur Pathogenese der akuten Aortendissektion beiträgt oder ob er eine Reaktion auf die enstandene Verletzung ist.

Die zweite Studie hat die Unterschiede in der räumlichen Verteilung des eNOS Proteins innerhalb der aufsteigenden Aorta von Patienten mit bikuspider Aortenklappe (BAV) zum Thema. Die BAV ist ein häufig auftretender, erblicher Defekt, der, im Gegensatz zur normalen trikuspiden Aortenklappe (TAV), die Ausbildung von nur zwei Aortenklappensegeln zur Folge hat. Die Erkrankung begünstigt die Enstehung von Aortenaneurysmen und Aortendissektionen, möglicherweise durch eine veränderte Hämodynamik und veränderte Scherkräfte, die durch die deformierte Aortenklappe hervorgerufen werden. Das eNOS Protein

wurde an vier verschiedenen Stellen innerhalb der Aorta von 14 Aneurysmapatienten mit BAV und 5 Aneurysmapatienten mit TAV quantifiziert. Die eNOS Proteinmenge unterschied sich signifikant zwischen verschiedenen Aortenbereichen in BAV, wohingegen die Unterschiede in TAV nicht signifikant waren. Zusätzlich wies die proximale Aorta von BAV Patienten signifikant weniger eNOS auf als das TAV Gegenstück. Diese Ergebnisse implizieren die räumliche Dysregulation der eNOS Expression in BAV, die wahrscheinlich durch die Veränderung der Scherkräfte hervorgerufen wird, ein Mechanismus der bekanntermaßen die eNOS Genexpression und Proteinaktivität reguliert. Das von eNOS produzierte Stickstoffmonoxid hat einen anti-apoptotischen Effekt auf das umliegende Gewebe, weswegen die Mengen von 35 Apoptose Proteinen mit Hilfe eines Proteome Profiler Arrays quantifiziert wurden, nachdem die Stichstoffmonoxidsynthese in kultivierten Aortenzellen inhibiert wurde. Danach war die cytosolische Menge der pro-apoptotischen Serinprotease HTRA2/Omi signifikant erhöht. Da das Protein normalerweise während des intrinschen Apoptosesignalweges aus den Mitochondrien in das Cytosol entlassen wird, scheint das von eNOS produzierte Stickstoffmonoxid einen inhibitorischen Effekt auf die intrinsiche Apoptose zu haben. Zusammengefasst deuten die Resultate dieser Studie auf die räumliche Dysregulation des eNOS Proteins in BAV hin, welche wahrscheinlich durch die veränderte Hämodynamik hervorgerufen wird und eine unregelmäßige Verteilung des Stickstoffmonoxids innerhalb der Aorta bewirkt. Der Verlust der anti-apoptotischen Eigenschaften des Stichstoffmonoxids in betsimmten Aortenarealen könnte die Apoptose von Aortenwandzellen zur Folge haben, ein Mechanismus, der erwiesenermaßen zur Pathogenese von Aneurysmen und Aortendissektionen in BAV beiträgt.

In der dritten Studie wurde die Expression der miRNA 208a in Gewebe von Patienten mit Vorhofflimmern quantifiziert. Das Vorhofflimmern stellt die häufigste Form der kardialen Arrhythmie dar und ist mit einem erhöhten Schlaganfallrisiko verbunden. Anhaltenes Vorhofflimmern kann sich selbst durch die elektrische und strukturelle Umgestaltung der Vorhöfe aufrechterhalten, weswegen die Krankheit entsprechend ihrer Dauer klassifiziert wird. Die herzspezifische miRNA 208a wurde in Gewebe vom linken Herzohr von 2 Patienten mit paroxysmalen, 10 Patienten mit persistierenden und 7 Patienten mit lang anhaltendend persistierenden Vorhofflimmern quantifiziert.

Die miRNA Expression war in Gewebe von lang anhaltend persistierendem Vorhofflimmern, im Vergleich zu persitierenden Vorhofflimmer, signifikant erniedrigt, was einen Rückgang über den Verlauf der Arrhythmie nagelegt. Die miRNA 208a wird mit dem *Myh6* Gen koexprimiert, welches die schnelle α Isoform der schweren Myosinkette kodiert. Es ist bekannt, dass bei anhaltendem Vorhofflimmern das Umschalten von der Expression des schnellen α Myosins auf die langsame β Isoform erfolgt und der Rückgang der miRNA 208a Expression könnte nur ein Nebenprodukt dieses Ereignisses sein. Andereseits könnte miRNA 208a auch von bedeutung für die Pathogenese des Vorhofflimmerns sein, da Studien mit transgenen Mäusen auf eine Funktion der miRNA in der Reizleitung des Herzens und der Arrhythmogenese hindeuten.

7. Complete List of Publications

7.1 Peer-reviewed Papers

1. Belge G, Radtke A, Meyer A, Stegen I, Richardt D, Nimzyk R, Nigam V, Dendorfer A, Sievers HH, Tiemann M, Buchwalow I, Bullerdiek J, Mohamed SA. Upregulation of the high mobility group AT-hook 2 gene in acute aortic dissection is potentially associated with endothelial-mesenchymal transition. Histol Histopathol. 2011; 26: 1029-1037.

2. Markowski DN, Helmke BM, Radtke A, Froeb J, Belge G, Bartnitzke S, Wosniok W, Czybulka-Jachertz I, Deichert U, Bullerdiek J. Fibroid explants reveal a higher sensitivity against MDM2 inhibitor nutlin-3 than matching myometrium. BMC Womens Health. 2012; 12: 2.

3. Mohamed SA, Radtke A, Saraei R, Bullerdiek J, Sorani H, Nimzyk R, Karluss A, Sievers HH, Belge G. Locally different endothelial nitric oxide synthase protein levels in ascending aortic aneurysms of bicuspid and tricuspid aortic valve. Cardiology Research and Practice. 2012; 2012: 8.

4. Mohamed SA, Noack F, Schoellermann K, Karluss A, Radtke A, Schult-Badusche D, Radke PW, Wenzel BE, Sievers HH. Elevation of matrix metalloproteinases in different areas of ascending aortic aneurysms in patients with bicuspid and tricuspid aortic valves. The Scientific World Journal. 2012; 2012: 7.

5. Radtke A, Hanke T, Yan J, Godau B, Cordes J, Nigam V, Sievers HH, Mohamed SA. MicroRNA 208 in atrial fibrillation. Journal of Clinical & Experimental Cardiology. 2014; 5: .

- The first two authors in publication 1., 3. and 5. contributed equally

7.2 Oral presentations

Radtke A, Mohamed SA. Aorta and aortic disease. Workshop „Kardiale Stammzelltherapie und Tissue Engineering“ Vilm 2010

7.3 Poster presentations

1. Radtke A, Belge G, Nimzyk R, Sievers HH, Bullerdiek J, Mohamed SA. Analysis of the endothelial nitric oxide synthase protein levels in different areas of thoracic aortic aneurysms. GFH Tagung 2010

2. Belge G, Radtke A, Meyer A, Stegen I, Richardt D, Nimzyk R, Nigam V, Dendorfer A, Sievers HH, Tiemann M, Bullerdiek J, Mohamed SA. Overexpression of *HMGA2* in acute aortic dissection may lead to EndMT of *vasa vasorum* endothelial cells. GFH Tagung 2011

3. Belge G, Radtke A, Paul A, Sievers HH, Bullerdiek J, Mohamed SA. Fibulin 1 as a potential biomarker in acute aortic dissection. GFH Tagung 2011

4. Radtke A, Belge G, Bullerdiek J, Sievers HH, Mohamed SA. The impact of eNOS inhibition on aortic endothelial cell apoptosis. GFH Tagung 2011

5. Belge G, Radtke A, Bullerdiek J, Sievers HH, Mohamed SA. The endoglin levels after treatement aneurysmal vascular smooth muscle cells of patients with bicuspid with losartan. GFH Tagung 2012

8. Abbreviations

°C	Degree Celcius
αMHC	Alpha myosin heavy chain
βMHC	Beta myosin heavy chain
ΔΔCT	Delta delta cycle threshold
µg	Microgram
µl	Microlitre
µm	Micrometer
A	Adenine
AAD	Acute aortic dissection
ACTA2	Alpha actin 2
ACTB	Beta actin
AF	Atrial fibrillation
aHTN	Aterial hypertension
ANOVA	Analysis of variance
AoV	Aortic valve
BAV	Bicuspid aortic valve
Bax	BCL2-associated X protein
BCA	Bicinchoninic acid
Bcl2	B-cell CLL/lymphoma 2
BMI	Body mass index
BSA	Bovine serum albumin
C	Cytosine
C. elegans	Caenorhabditis elegans
Ca^{2+}	Calcium ion
CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit
CACNB1	Calcium channel, voltage-dependent, beta 1 subunit
CAD	Coronary arterial disease
CD-34	CD34 molecule
cDNA	Complementary DNA
CDKN1A	cyclin-dependent kinase inhibitor 1A
CHADS2	Congestive heart failure/ hypertension/ age/ diabetes mellitus/ stroke
CHF	Chronic heart failure
cm^2	Square centimetre
CO_2	Carbon dioxide
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ESC	European Society of Cardiology
et al.	et alii
f	Female
FADD	Fas-associated via death domain
FAM	6-carboxyfluorescein
FCS	Fetal calve serum
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA5	GATA binding protein 5
GJA1	Gap junction protein, alpha 1
H	Hour
H&E	Hematoxylin and eosin stain
HAEC	Human aortic endothelial cells
HCl	Hydrogen chloride
HIF-1a	Hypoxia-inducible factor 1 alpha
HMGA2	High mobility group AT-hook 2
HMOX1/HO-1	Heme oxygenase 1

HMOX2/HO-2	Heme oxygenase 2
Hoxa1	Homeobox A1
HSP	Heat shock protein
HTRA2/Omi	HtrA serine peptidase 2
I_{Ca}	Inward C-type Ca^{2+} current
IgG-AP	Immunoglobulin G with alkaline phosphatase
iNOS	Inducible nitric oxide synthase
KCNJ2	Potassium inwardly-rectifying channel, subfamily J, member 2
KCNN3	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3
kg	Kilogram
K-Ras	Kirsten rat sarcoma viral oncogene homolog
LA-D	Left atrium diameter
Let-7	Lethal-7
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
LV-EF	Left ventricle ejection fraction
m	Metre
M	Molar
m^2	Square metre
mg	Milligram
$MgCl_2$	Magnesium chloride
Min	Minute
miRNA	Micro RNA
MKI-67	Marker of proliferation Ki-67
ml	Millilitre
mM	Millimolar
mm	Millimetre
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Marfan syndrome
MV	Mitral valve
MYH11	Myosin, heavy chain 11
Myh6	Myosin, heavy chain 6
Myh7	Myosin, heavy chain 7
Myh7b	Myosin heavy chain 7b
NaCl	Sodium chloride
NBT/BCIP	Nitro blue tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate
ng	Nanogram
Nkx2-5	NK2 homeobox 5
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NYHA	New York Heart Association
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDIA2	Protein disulfide isomerase family A, member 2
pH	Potentia hydrogenii
PITX2	Paired-like homeodomain 2
PON2	Paraoxonase 2
R^2	Coefficient of determination
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNU48	small nucleolar RNA, C/D box 48
RNU6B	RNA, U6 small nuclear 6
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SNAI1	Snail family zinc finger 1
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA

SPSS	Statistical package for the social sciences
STJ	*S*inotubular junction
SV40	Simiam virus 40
T	Thymine
TAA	Thoracic aortic aneurysm
TAV	Tricuspid aortic valve
TBS-T	Tis buffered saline –Tween 20
TGFbRII	Transforming growth factor-beta receptor type 2
TGF-β/TGFB	Transforming growth factor, beta 1
Tie-2	Tunica internal endothelial cell kinase 2
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TNFSF6	TNF superfamily, member 6
TRAIL	TNF-related apoptosis-inducing ligand
TV	Tricuspid valve
U	Unit
UFD1L	Ubiquitin fusion degradation 1 like
UTR	Untranslated region
V	Volt
VD	Vascular disease
vs	Versus
VSMC	Vascular smooth muscle cells
xg	Standard gravity
XIAP	X-linked inhibitor of apoptosis
ZFHX3	Zinc finger homeobox 3

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11. Declaration/Erklärung

I hereby declare that:

1. I have written the thesis without the unauthorised aid of others.
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Bremen, 17.11.2014

Mark Arlo Radtke