Elucidating the pathogenesis underlying bicuspid aortic valve disease using new disease models

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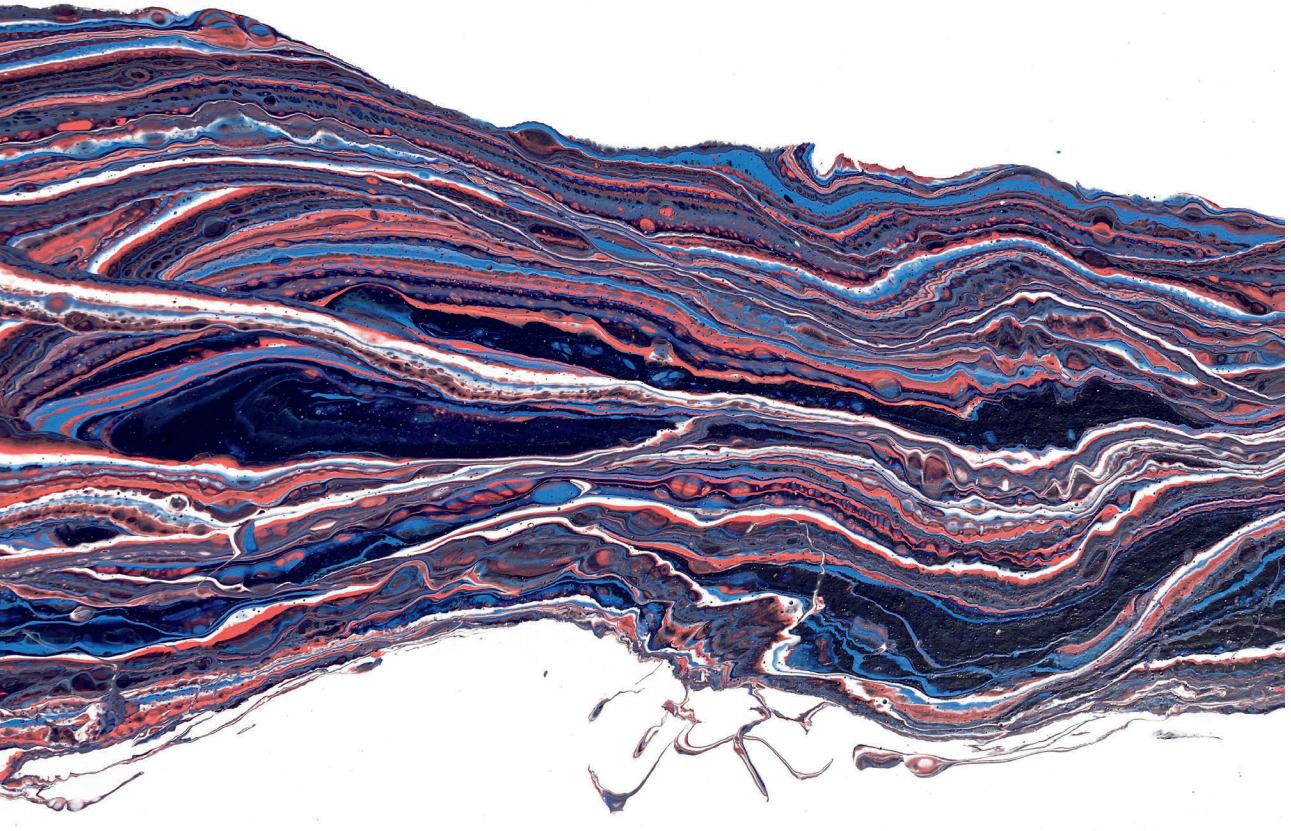
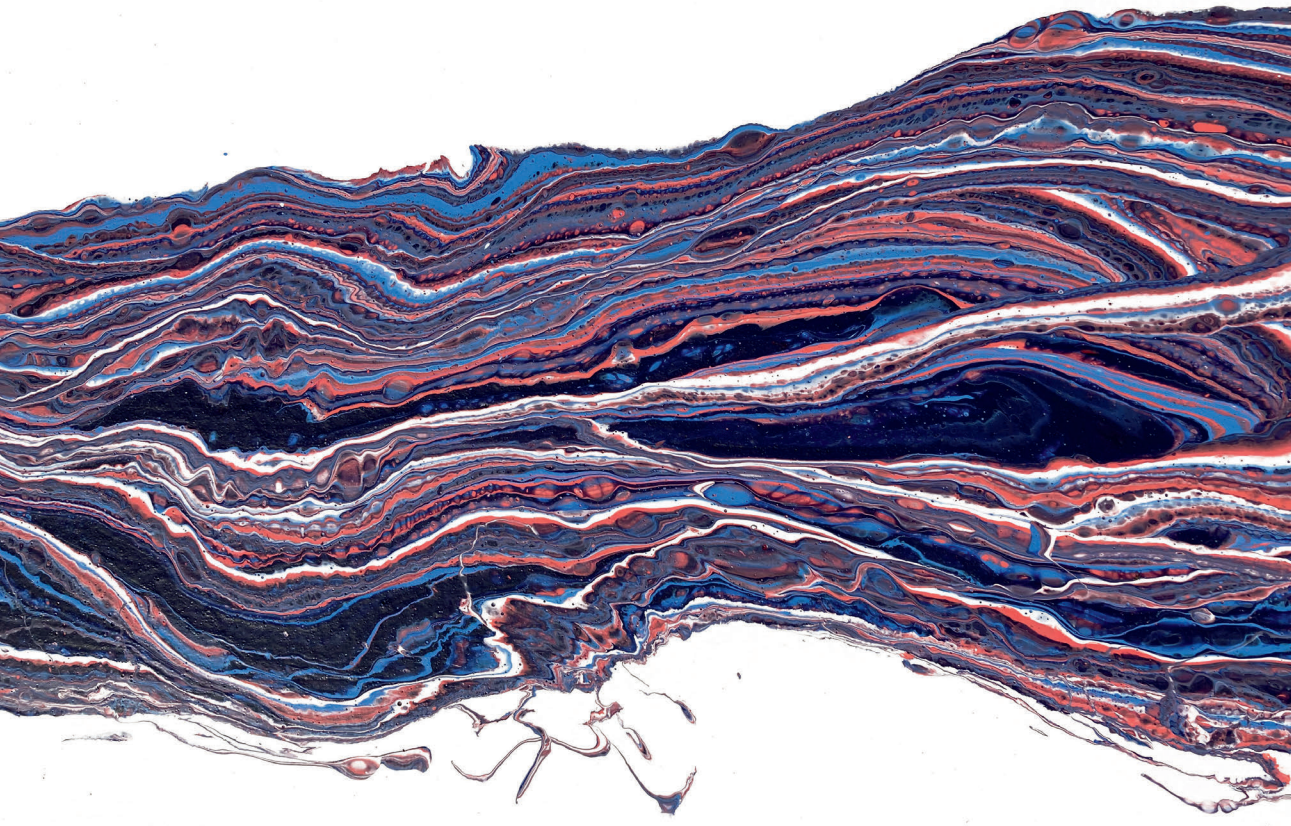


Chapter 10

General discussion



Bicuspid aortic valve (BAV) is the most common congenital heart defect and in patients with a BAV the prevalence of calcific aortic valve disease (CAVD) and thoracic aortic aneurysms is increased [1,2]. To date, the pathogenesis of BAV as well as the associated CAVD and aneurysm formation are not fully understood. Currently, the only available treatment options for CAVD or aortic aneurysm are to replace or repair the malfunctioning aortic valves and/or the ascending aorta/aortic root. Therefore, there is an urgent need to develop a treatment that will delay, prevent and possibly even reverse CAVD and aortic aneurysm formation. This requires an increase in our understanding of the mechanisms behind BAV and the related pathologies.

Different factors have hampered research aiming to understand the pathogenesis of BAV and disease progression of CAVD and aortic aneurysm in BAV patients. BAV is usually only diagnosed after patients experience health problems related to the aortic dilation or CAVD. Therefore, the initiation of the dilation or CAVD as well as the formation of the BAV itself is difficult to study in humans. Moreover, tissue biopsies from the aortic valves or the aortic wall are not available unless the patient receives valvular and/or aortic replacement surgery. Finally, although genetic studies have identified multiple gene mutations and genetic variants associated with BAV, there is not (yet) one common genetic profile associated with all BAV patients. All these factors make research on the aetiology of BAV rely heavily on experimental models and end-stage materials.

Much of the research on BAV aneurysm formation is focussed on the media and the function of smooth muscle cells (SMCs), because the most obvious pathological remodeling in aortic dilation often occurs in the media. The endothelial cells (ECs) lining the aortic wall form only a small fraction of the cells present in the vessel wall and, consequently, the role ECs play in the development of BAV and the associated pathologies is rarely studied. Although the endothelial layer is thin, multiple cardiovascular diseases have been shown to start with EC dysfunction and many disease models show the impact EC dysfunction can have on the vessel wall.

Therefore, the aim of this thesis was to study the underlying pathogenesis of aortic valve calcification and aortic dilation in BAV patients using different (novel) disease models and increase understanding of the role of ECs in these processes.

Novel models for development of CAVD; the potential for BAV research

Aortic stenosis, the most severe stage of CAVD, can lead to cardiac hypertrophy and often requires aortic valve replacement surgery to prevent heart failure. But even after valve replacement surgery, patients may still require life-long medi-

cation and monitoring. BAV CAVD patients require valve replacement surgery at a younger age than TAV CAVD patients, increasing the possibility that BAV patients require a second replacement surgery later in life. To prevent this lifelong impact of valve replacement surgery, CAVD disease progression should be halted or even reversed or prevented. To achieve this a detailed knowledge of the pathological mechanisms of CAVD from initiation until the end is required.

To study the initial stages of CAVD we described an *ex vivo* calcification model to study CAVD in the native configuration of the valves (chapter 6). We adapted the culture protocol of the previously developed miniature tissue culture system (MTCS), in which mouse aortic valves maintain their natural position in the heart in a controlled setting [3]. We were able to model different forms of calcification in the aortic valve using two different media, inorganic phosphates (PI) or osteogenic medium (OSM), in the MTCS. Most strikingly, our data showed that when using medium containing PI, but not when containing OSM, calcification can be induced in the aortic valves of wild-type (WT) mice when cultured *ex vivo*, while both OSM and PI induced calcification *in vitro.* These differences between *in vitro* and *ex vivo* calcification and between PI or OSM induced calcification reiterate the importance of the complex, local environment of the valves in the development of CAVD.

The *ex vivo* cultured leaflets expressed markers of osteogenic differentiation such as COX2 and RUNX2. Especially COX2 colocalized with calcified nodules in the valves of PI cultured hearts. In addition, cartilage formation was observed in the root of the aorta in both PI and OSM *ex vivo* culture conditions, indicating the presence of different forms and mechanisms of calcification in the *ex vivo* cultured aortic valves demonstrating the value of 3D modelling [4,5]. Interestingly, in contrast to previously published findings, the addition of dexamethasone abrogated the formation of calcification induced by the PI treatment [6]. Moreover, alkaline phosphatase (ALP) expression in the valves was also prevented in all conditions by the addition of dexamethasone. In addition, dexamethasone has been widely used as an adjuvant in mesenchymal cell culture medium to induce calcification in many different cells including valvular interstitial cells (VICs) [7,8]. The observed discrepancy with our study could be due to the use of different type of cells, distinct protocols, and differences between *in vitro* versus *ex vivo cultures.*

Tissue stiffness, extracellular matrix (ECM) and cellular composition of the aortic valves as well as the flow and blood composition to which they are exposed are important determinants in the valvular calcification process [9-12]. The culture model described in chapter 6 offers a novel method to study the initial stages of valvular calcification in the native environment of the aortic valve.

It is one of the very few methods allowing to study CAVD in an environment closely resembling the aortic valves *in vivo*. Complementing this model to unravel disease progression is a diet-based-method (high cholesterol, phosphate and calcium, low vitamin D3) that is able to induce calcification in the valvular commissures in WT mice [13]. Moreover, two-photon excited fluorescence (TPEF) microscopy allows early detection of CAVD progression by providing quantitative measurements that correlate with calcium deposition, collagen remodeling and osteogenic differentiation [13]. This new *in vivo* model has great potential for the progression research of CAVD in BAV if the pro-calcific diet can also induce calcification in different genetic mouse models for BAV [14-17]. The main differences between the *ex vivo* MTCS and the diet-induced mouse models are the method of induction of CAVD and the type of flow the valves are exposed to. While the diet-induced CAVD animal model makes use of mice and the physiological valvular flow, the MTCS culture model allows the study of different types of flow on the aorta and aortic valves. Furthermore, *ex vivo* modelling allows for more manipulation compared to (ethically acceptable in) live animal models.

In addition, an interesting abstract was recently published demonstrating organ-on-a-chip modelling of CAVD in a complex co-culture of VICs and valvular endothelial cells (VECs) [18]. Innovations like this will greatly increase the suitability of cell culture in research towards CAVD in the future. If cells from BAV patients would be used in this model, the underlying mechanisms in CAVD pathogenesis in BAV may be further elucidated.

A role for FHL2 in CAVD and BAV associated aortic dilation

FHL2 is a scaffold protein known to be involved in the cardiovascular response to changes in homeostasis and other triggers. For example, FHL2$^{-/-}$ mice have an exaggerated cardiac hypertrophic response to β-adrenergic stimulation and an increased SMC proliferation rate after carotid artery ligation, exacerbating vascular lesion formation [19,20]. Moreover, FHL2 is known, amongst others, for its involvement in calcification processes [21,22]. Interestingly, dexamethasone was shown to induce osteogenic differentiation of stem cells in vitro via FHL2/β-catenin-mediated transcriptional activation of RUNX2 [7].

FHL2 in CAVD

To investigate the role of FHL2 in cardiovascular calcification, we studied the role of FHL2 in calcification of the aortic valves *ex vivo* (chapter 7). *Ex vivo* cultured valves from FHL2$^{-/-}$ mice with PI did not show a significant difference in the amount of calcification. However, the location of the calcified nodules

was significantly different between FHL2$^{-/-}$ and WT aortic valves. In the FHL2$^{-/-}$ valves, the calcification nodules located more towards the aortic side whereas in the WT valves the calcification was located mostly at the ventricular side of the valve. Our data shows that FHL2 has the potential to impact valvular calcification. These spatial specific results could not have been discovered with *in vitro* modelling nor in human aortic valve tissues obtained during valve replacement surgery, demonstrating the unique results that can be achieved with this model.

Our results demonstrate that the effect of FHL2 on formation of calcified nodules is different between the aortic and ventricular side of the leaflet. Since FHL2 is expressed by different cell populations throughout the body and is involved in many different processes, targeting FHL2 systemically would likely have numerous side-effects [19,20]. One study reported that it is possible to deliver a drug in specifically the aortic valves using a drug-eluting balloon during valvuloplasty [23]. Further development of similar procedures may allow targeting of FHL2, or other possible targets such as miRNA's, specifically in the aortic valves and can provide innovative treatment options for CAVD in the future [24]. However, our results suggest that targeting of FHL2 may require not only a delivery method that is valve specific, but also one that is specific for one side of the leaflet. Therefore, miRNA's that target multiple proteins involved in CAVD may be a more realistic treatment option.

FHL2 and the aorta

Because FHL2 plays a role in the differentiation status and proliferation rate of SMCs, we hypothesized that FHL2 plays a role in the aortic medial degeneration that occurs in aortic aneurysm formation (chapter 8) [19,25,26]. We found that FHL2 is increased in dilated compared to non-dilated TAV aortas but not in dilated compared to non-dilated BAV aortas. Especially in the middle of the media, in the pathological absence of αSMA, FHL2 expression was high. Furthermore, we found large variations between patient samples in the amount of FHL2 present in the aortic wall, reiterating significant heterogeneity in patients with a BAV. Moreover, we found that FHL2 is secreted by SMCs in extracellular vesicles (EVs) and is present in human plasma as demonstrated by Western blotting. The secretion of FHL2 into plasma increase its usefulness as potential biomarker. However, to date, we were unable to find a difference in plasma FHL2 levels between TAV controls and BAV patients with and without aortic dilation. Although our data illustrates that aortic remodeling is a local response, it may be possible to monitor these local processes from a distance. It has been shown that EVs secreted by SMCs with a contractile phenotype have a distinctly different content from EVs secreted by synthetic SMCs [27]. If these EVs can be isolated

and analysed from plasma, using SMC specific markers, similar to the way in which cancer specific EVs are studied in plasma, FHL2 in these EVs may prove to be suitable as a marker for aortic dilation in TAV [28].

To further understand the role of FHL2 in the vasculature and because FHL2 is also involved in inflammation, we studied the role of FHL2 in SMCs and vascular inflammation (chapter 9). We found that both systemic and local vascular inflammation is increased in FHL2$^{-/-}$ mice after carotid artery ligation. In addition, FHL2$^{-/-}$ SMCs secreted increased amounts of chemokines and cytokines, which was brought back to baseline when FHL2 levels were exogenously increased. Finally, we show that blocking the NFκB pathway reverts the inflammatory phenotype of FHL2$^{-/-}$ SMCs. These results indicate that FHL2 inhibits the inflammatory response of vascular SMCs through attenuating NFkB-signaling.

We found that FHL2 decreases inflammation in aortic SMCs and that there is an increase in FHL2 expression in dilated TAV aortas. Moreover, TAV dilated aortas have an increased inflammatory state compared to dilated aortas of BAV patients [29]. Possibly, FHL2 is present in the TAV dilated aortic tissue to dampen the inflammatory signaling cascade. However, the effect of FHl2 expression in TAV and BAV aortic wall remains to be discovered. Since FHL2 is a scaffolding protein, it affects many pathways simply by bringing different proteins together, and its function is largely context and cell type dependent. Therefore, the proteins FHL2 interacts with in TAV or BAV aortas will determine the downstream signaling. Furthermore, localization and expression levels of FHL2 might determine the outcome in specific cell type in different diseases (demonstrated by multiple seemingly contradicting articles [30-33] and our own results on the locally different effects in response to PI during *ex vivo* culture in FHL2$^{-/-}$ valves). Many proteins are differently expressed between TAV and BAV (dilated) aorta [29,34,35]. Therefore, the signaling pathways in which FHL2 is involved might be very different between TAV and BAV, even if the amount of FHL2 expression is similar. Of course, this holds true for all comparisons in which FHL2 is not the only protein that is differently expressed, hampering direct conclusions from patient-control experiments. Keeping this in mind whilst studying FHL2 will lead to a better understanding of how FHL2 affects which pathways in different contexts. Future research will hopefully allow completion of the equation and help to understand 1) why FHL2 expression is increased in TAV aortic dilation but not in BAV aortic dilation, and 2) what the effects are of this increase.

Endothelial cells in BAV and aortic dilation

Most research to understand BAV related aortic dilation is focussed on the SMCs and medial degeneration, as the media is the location of the major pathology.

However, the vessel wall is more than only SMCs. As detailed in Chapter 2, ECs play a major role in vascular integrity and remodeling and are under-investigated in the pathogenesis of BAV. Consequently, there are only few experimental models available to study EC function of BAV patients. Patient derived endothelial cells would be a suitable model to gain more insight in the role of the endothelium in BAV. One of the major advantages of patient specific ECs is that the (epi)genetics and resulting altered mechanisms can be studied *in vitro*. This is especially relevant for modelling diseases in which not one gene is responsible for the pathology, and genetically manipulated mouse and cell models are not able to fully model the genetic background, such as BAV [36].

An in vitro model of BAV patient ECs: ECFCs

Isolating aortic endothelial cells relies on the availability of aortic tissue. Endothelial colony forming cells (ECFCs) are cells that can be isolated from the mononuclear cell fraction of blood and have been used as a patient specific *in vitro* model for a range of different diseases involving the cardiovascular system such as vasculitis, pulmonary arterial hypertension, and diabetes, providing insights in disease development and mechanism [37-40]. Given the important role of EC function in vascular homeostasis, we aimed to investigate EC function in BAV in Chapter 4. Therefore, we isolated and characterised ECFCs from BAV patients and healthy controls. Relating the outgrowth and proliferation of ECFCs to patient characteristics, we observed a striking decrease in efficiency of successful ECFC isolation in BAV patients with a dilated aorta. In the ECFCs that were successfully isolated, proliferation rate and cell size were similar but there was a decrease in migratory behaviour of ECFCs from BAV patients when compared to TAV controls. A recent study shows that, besides a smaller amount of calcification in CAVD between younger BAV females and older TAV males, there is no difference between the amount of calcification in CAVD between TAV and BAV patients [41]. However, the increased prevalence of CAVD in BAV patients lead us to hypothesize that the calcification experiment would show an increased amount of calcification in BAV ECFCs compared to TAV ECFCs. Although BAV ECFCs were able to spontaneously develop calcified nodules, the calcification response to osteogenic medium was higher in TAV ECFCs than BAV ECFCs. It is possible that ECs are not involved in the increased prevalence of CAVD in BAV or that ECFCs do not accurately reflect the BAV EC response to osteogenic stimulation. Another possibility, however, is that the used method of calcification is not suitable to study the differences between BAV and TAV calcification processes. Both PI and the OSM medium used for mVICS and the MTCS-CAVD model were not able to induce calcification in the ECFCs. This also relates to the observation

in chapter 6 that different protocols stimulate calcification in different ways and will thereby lead to different conclusions. Unfortunately, protocols inducing a calcification process in ECFCs are sparse, limiting the study of this phenomenon in current patient-specific experimental models. Therefore, further optimization of protocols (e.g.: longer incubation, higher concentration, etc.) using PI, OSM or similar is needed to study calcification in ECFCs to further understand the role of ECs in CAVD development in BAV patients.

Our results in chapter 5 show that inflammatory stimuli IL-1β and TNF-α induce endothelial to mesenchymal transition (EndoMT) in primary ECs, which become prone to undergo osteogenic differentiation in response to BMP-9, a potent ligand of the BMPR2 receptor in ECs. We identified BMPR2 downregulation as a key event in this process, which leads to decreased JNK activation, thereby enhancing BMP-9-induced mineralization. Interestingly, BMP9 can bind ALK2 and deficient signaling via ALK2 has been shown to cause BAV in mice [42]. Therefore, it may be possible and interesting to further understand the BAV EC calcification by pre-treating the ECFCs with an inflammatory stimulus. This is further supported by our result showing that PiT2, a type III sodium-dependent phosphate transporter is increased in TAV but not in BAV ECFCs after TNFα stimulation.

The translation of *in vitro* results to *in vivo* effects is often difficult and this certainly applies to ECFCs as well, caused by, amongst others, the difference between the simple environment of a culture dish compared to the complex *in vivo* homeostasis. The results of the *in vitro* migration assay are an example of this complex issue. Although the *in vitro* scratch migration assay is meant to mimic a wound healing response, there was no correlation between migration of ECFCs *in vitro* to the decreased wound healing observed *in vivo* in patients with diabetes [38]. Therefore, although we observed an increase in the *in vitro* migration rate of BAV ECFCs compared to TAV ECFCs, we are unable to conclude what effect this may have on *in vivo* ECFC or EC behaviour. More research will increase knowledge on the association between disease phenotypes with specific altered responses of ECFCs and improve the interpretation of ECFC *in vitro* response to *in vivo* functioning.

Intriguingly, resident ECFCs have recently been found in the developing, neonatal lungs, and ECFC dysfunction was related to the development of congenital lung diseases [43-46]. Although the role of ECs in aortic valve formation is well-known, the involvement of ECFCs is not explored. One might speculate that ECFCs are also involved in valvulogenesis and therefore ECFC dysfunction is potentially involved in the formation of congenital valvular diseases, such as BAV, during development.

Aortic ECs of BAV patients

The limitations of 2D cell culture in modelling a complex environment also affects research involving ECs. In their native environment, aortic ECs are exposed to amongst others flow, immune cells and vascular SMCs [47]. To increase our understanding of EC functioning in BAV we studied aortic ECs in their complex, natural environment by histologically characterizing ECs in aortic samples from BAV patients (chapter 3). The tissue samples were obtained from the aortic wall at two locations: the 'jet' side and the 'non-jet' side. This allowed a study of the effect caused by different flow patterns in the BAV aorta on endothelial activation. One of the strengths of this approach is that ECs from different aortic locations can be compared within one patient. Therefore each patient is its own control, minimizing patient variabilities and taking confounding factors out of the equation that may occur by grouping BAV patients together, such as different genetic causes, pathogenic mechanisms, and disease progression. Histological analysis demonstrated that ECs at the non-jet side of the aorta showed a trend towards higher expression of inflammatory, EndoMT and proliferation pathways compared to the ECs on the jet side, indicating a more active EC phenotype at the non-jet side. These data are in line with research showing that the non-jet side of the BAV aorta is exposed to oscillatory flow and studies demonstrating that ECs are activated by oscillatory flow [48-51].

Although this study makes use of a unique set of samples, one aspect that would be interesting to study, but is limited in this dataset, is the comparison of EC activation to a healthy control. Mouse models of BAV could be used for this comparison, allowing the study of flow on ECs and compare BAV to TAV [15,52]. In addition, mouse models would allow the study of ECs in a less diseased aorta compared to the end-stage disease tissues obtained after surgery. Especially since many mouse models of BAV do not show a 100% penetrance of the phenotype, the TAV mutant mice could serve as a genetic control. Unfortunately and intriguingly, in literature we could not confirm whether BAV in mice alters the aortic flow similarly to BAV in humans, indicating that there may be untapped potential in the use of these models. Since flow can be manipulated in the MTCS, this *ex vivo* model may also provide interesting insights into the relation between BAV, flow and aortic remodeling.

In addition, we found that there are large variations in histological morphology in diseased human aortic tissues. Chapter 3 briefly describes the local differences of vWF expression, but also stainings for αSMA showed great diversity within one tissue sample. Figure 1 shows data from three locations in one tissue sample. Strikingly different conclusions can be drawn from these three graphs and will be drawn if the localized diversity of the aorta is not taken

into consideration. This does not count for all proteins as can be appreciated for our vWF expression showing the same average expression level differences between patient groups as when measured at one specific location. Local differences should be taken into consideration when designing experiments and the methods used and should fit the research question. Depending on the research question a certain characteristic can be measured at multiple locations within one sample (studying the general quality of the tissue) or a specific location can be studied for all samples (studying local processes).

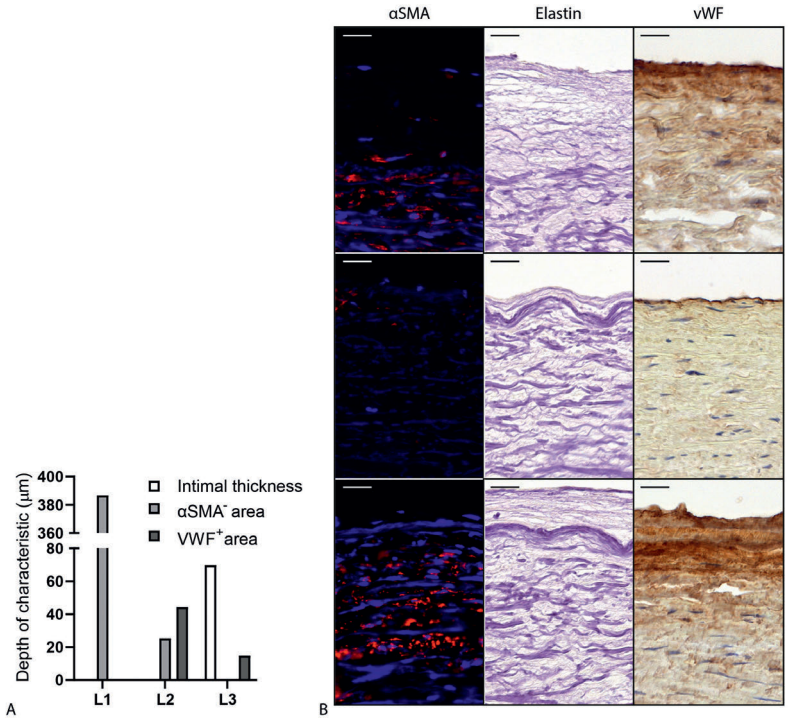


Figure 1. Variation within aortic tissue samples. Illustrative graph (A) and images (B) of one single patient sample in vWF and αSMA expression and intimal thickness. Scalebar is 20 μm.

Future perspectives

The ultimate aim of BAV research is the development of a non-invasive treatment preventing or reversing CAVD and aortic dilation. Moreover, this needs to be combined with a method to detect CAVD and aortic dilation in an early stage of the disease. To be able to develop these treatment options, the initiating and

progression occurring in these pathologies need to be well understood. The difficulty of BAV is that the initiation and development of a BAV can hardly be studied in humans. Furthermore, the molecular characteristics of the valves and the aorta can hardly be studied in the early stages of CAVD and aortic dilation. Therefore, accurate experimental models are required.

The MTCS is an interesting new model to study the role of flow on the valves and aorta in BAV and TAV mouse models. Future studies using the MTCS may help in understanding the role of flow, specific proteins and treatments in BAV and TAV aortic remodeling and CAVD, in addition to the other diseases that can be studied in the MTCS.

To understand the cellular functioning of BAV ECs, VICs and SMCs, another model that could be used is a combination of *in vitro* models such as valve- and vessel-on-a-chip with patient specific cells such as ECFCs and iPSCs derived vascular cells [18,53]. These models allow detailed analysis of the different molecular pathways involved in processes such as SMC phenotype, ECM maintenance and calcification. Moreover, patient-specific models can be used to study different treatments targeting these processes.

ECFCs provide a novel patient-specific cell model to study pathological processes in BAV. While iPSCs can also be differentiated towards ECs, ECFC culture does not require a differentiation stage. This may be preferred in studying BAV EC characteristics since many mutations found in BAV are involved in cellular differentiation, possibly affecting the EC phenotype of differentiated BAV but not TAV iPSCs. It has already been shown that BAV iPSCs have a defect in differentiation towards a SMC [54]. This does illustrate that iPSCs can provide valuable information on the differentiation of BAV ECs and thereby aid in understanding of the formation of BAV during valvulogenesis.

The use of patient-specific material in BAV research should, however, be accompanied by the awareness that BAV is not one disease but a pathology which can be caused by a myriad of pathogenic mechanisms [17,55]. In our study using ECFCs we were able to generate significant results with a small sample size, however, future studies likely require a larger sample size to generate more detailed results due to the non-homogenous population that characterizes BAV.

All research in experimental models requires translation to patients. Because of the lack of tissue samples in BAV during the early pathogenesis, the patients can be monitored using advanced imaging techniques and detection of key factors in the blood. To detect CAVD and aortic dilation in an early stage of the disease, biomarkers are and should be studied. Understanding the pathological processes will help targeting the search for a biomarker. FHL2 may have some potential to serve as a biomarker. Especially in TAV dilation FHL2 in SMC spe-

cific EVs may be used to monitor the local aortic medial degeneration. Although the study of correlations between plasma samples and the aortic histology of patients may provide interesting results, long-term follow-up studies are required to validate plasma samples or EVs as a biomarker predicting aortic pathology. Furthermore, FHL2 is an interesting protein to increase understanding of CAVD, flow and tissue stiffness. To understand the role of FHL2 under regular flow conditions the diet-induced CAVD mouse model may provide interesting insights [13]. To understand the role of FHL2 in aortic dilation, BAV and TAV, patient specific cells in different co-culture and flow experiments, such as the valve- and vessel-on-a-chip models can be used.

The combination of new insights using accurate experimental models with patient samples and data will increase the understanding of the BAV pathogenesis that will, over time, turn into new and better treatment options for BAV patients.

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