Developmental cell lineage dynamics in Bicuspid Aortic Valve disease

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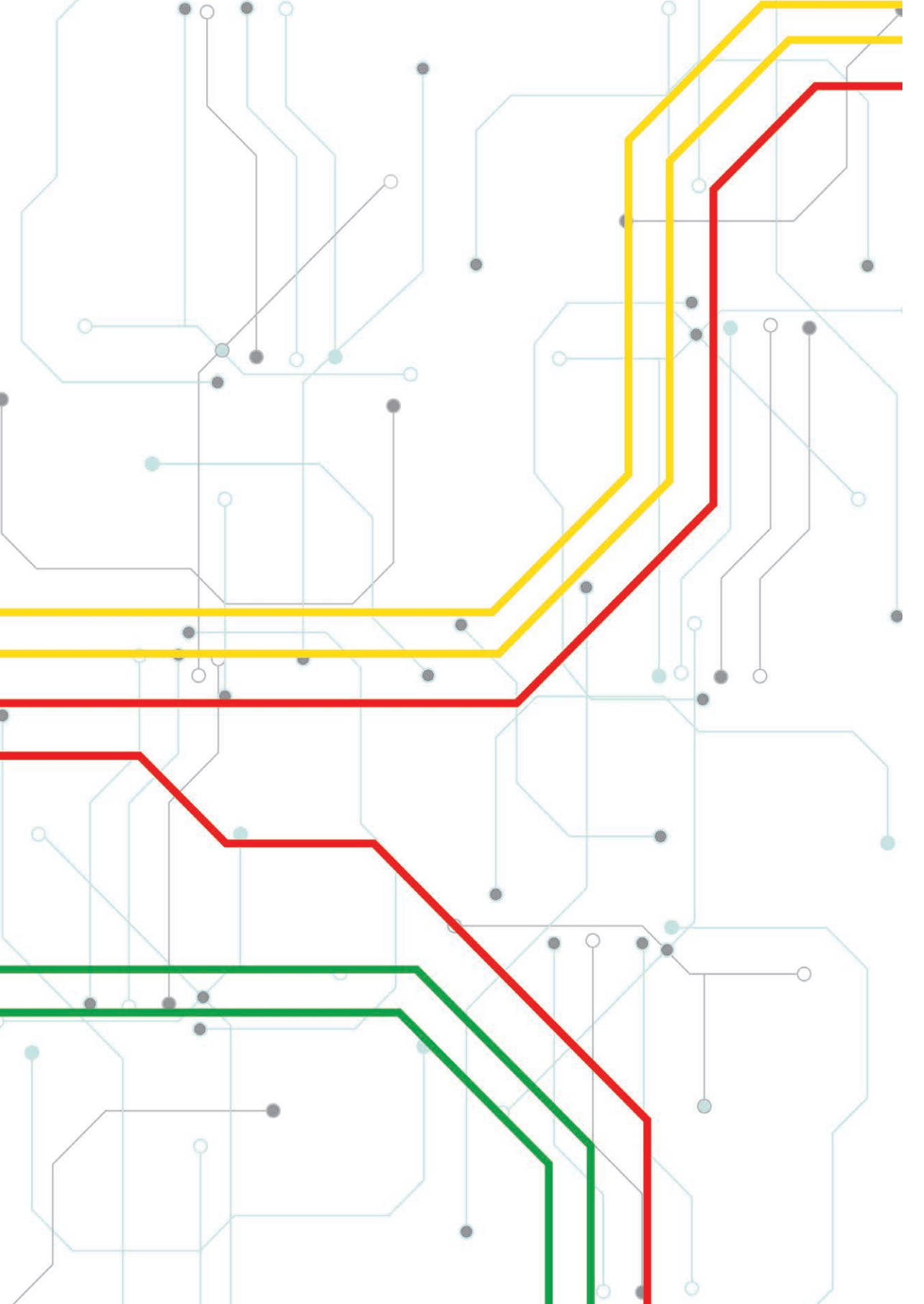

Chapter 7

Discussion and Future perspectives

7.1 Discussion

The studies presented in this thesis aimed to explain the increased susceptibility of BAV related aneurysms. Our results demonstrate that the developmental processes that give rise to BAV and BAV-related aortopathy can indeed develop from a common cell lineage anomaly that influences cell lineage contribution to the developing valve. Our studies can explain the clinical observations of increased susceptibility among patients of BAV and related aortopathy as our model shows that deviations in cellular contribution of the neural crest to the aortic valves and base of the aorta results in phenotypes that seem to support the observations in clinical patients. The value of these discoveries lies in the explanation of an deterministic model of disease progression. Knowledge of the fact that neural crest cells can affect both the aortic valves as the aortic vessel walls may influence the scope of clinical intervention strategies and support decision making with logical reasoning and increased certainty.

The chapters presented within this thesis address BAV and BAV-related aortopathy as a consequence of cell lineage anomalies occurring during early cardiac development. To examine these developmental aberrations our laboratory primarily studied the *Nos3*$^{-/-}$ BAV mouse model. These mice develop BAV as a result of a genetic mutation resulting in defective NO signalling (*Lee et al., 2000*).

Whilst it is very common for researchers to study BAV using well described mutant mouse models of BAV, these models usually represent medical cases of familial BAV. Whether a BAV is familial can be determined by genetic linkage analysis of close relatives (*Cripe et al., 2004*). Familial BAV is often the result of a genetic mutation which propagate through families, such as BAV patients with the NOTCH1 mutation (*Garg et al., 2005*). Such genetic mutations result in a relative high abundance of BAV within a single family. Curiously, familial BAV only represents <10% of clinical BAV cases whilst in >80% of BAV patients there is no prior familial history of BAV (*Prakash et al., 2014*). These later group of patients can be classified as having a sporadic BAV. Performing linkage analysis on sporadic BAV cases is unfeasible given that there are no prior familial observations of BAV. To find a causal explanation for sporadic BAV patients, researchers have instead performed numerous genome wide association studies (GWAS). GWAS studies typically require large populations of patients and controls to attain meaningful results. This is because GWAS identifies indirect associations between the genuine risk variants and genotyped single nucleotide polymorphisms, rather than by direct identification of the causal variants (*Visscher et al., 2012*). Genetic association studies have been able to determine

novel polymorphisms in sporadic BAV patients such as variations in AXIN1/PDIA2 (*Wooten et al., 2010*), Endoglin (*Wooten et al., 2010*), EFGR (*Dargis et al., 2016*), GATA4 (*Yang et al., 2017*), PALMD (*Helgadottir et al., 2018*) and SMAD6 (*Gillis et al., 2017*). Yet, despite the detection of novel variants a common genetic pathway specific to BAV has not been found. Moreover, there often remain many BAV patients within such studies for which a causal genetic explanation remains elusive.

The observation that many sporadic BAV patients do not contain any known mutations for BAV, led to the theory of a non-congenital adaptive/acquired BAV phenotype. Adaptive BAVs are generally considered to result from a valvular adaptation to environmental stress. Evidence for adaptive BAVs can be found in patients with rheumatic heart disease (*Veinot, 2006*). Patients with rheumatic heart disease are generally born with tricuspid aortic valves but can develop fusion of the aortic leaflets in response to inflammation related to rheumatic fever (*Veinot, 2006*). These patients can therefore acquire a BAV phenotype without a genetic component. Nevertheless sporadic BAV patients usually do not suffer from rheumatic heart disease or any other prevalent diseases other than BAV when participating in research studies.

I however, regard that the inability to detect a genetic component in certain sporadic BAV cases is primarily a limitation of genetic association studies. To briefly explain, genetic association studies primarily rely on the premise that an equal phenotype would suggest an equal underlying genotype. That is, when comparing genetic variation within a given population of patients with a certain disease-trait to the genetic variation of the general "healthy" population without said disease-trait. Then that variation that is specific to the disease-trait must be significantly overrepresented within the "diseased" patient population and thus distinct from the genetic variation of the general "healthy" population. This study design will however fail to find meaningful genetic associations when: 1) The disease-trait may originate from a large combination of multiple genetic anomalies, as this could require an impossible number patients to test. 2) The disease-trait has a partial penetrance of less than 50%, which results in a situation where the healthy population could potentially also carry the genetic variation causal to the disease-trait. 3) The disease-trait is not genetic in origin but acquired through environmental stimuli. In the case of BAV there can be raised arguments for all three criteria. One generally recognized challenge with BAV are the large variation of genes which have been linked to BAV. Genetic variations in TGFBR2 (*Girdauskas, Schulz, et al., 2011*), NOTCH1 (*Garg et al., 2005*), ACTA2 (*Guo et al., 2007*), KCNJ2 (*Andelfinger et al., 2002*), MAT2A (*Guo et al., 2015*) SMAD6 (*Tan et al., 2012; Gillis et al., 2017*) FBN1 (*Pepe et al., 2014*), GATA4/5/6 (*Shi et al., 2014; Li et al.,*

2018; Xu et al., 2018) and NKX2.5 (*Qu et al., 2014*) have all been found to result in BAV in human patients. Furthermore gene defects such as, NOS3 (*Lee et al., 2000*), ROBO1/2 (*Mommersteeg et al., 2015*), ROBO4 (*Gould et al., 2018*), ALK2 (*Thomas et al., 2012*), MATR3 (*Quintero-Rivera et al., 2015*), HOXA1 (*Makki and Capecchi, 2012*), KROX20 (*Theron et al., 2015*) and TBX20 (*Cai et al., 2013*) are known to give rise to BAV in mice but thus far have not been linked to human patients. However, BAV models such as GATA5/5/6 (*Laforest, Andelfinger and Nemer, 2011; Laforest and Nemer, 2011*) and NKX2.5 (*Biben et al., 2000*) knockouts were also identified in mouse prior to discovery of their role in human BAV patients. These observations suggest an even richer palette of possible gene defects to result in BAV for humans than that which is currently described. More interesting is the general acceptance of the limited penetrance of BAV despite our poor understanding regarding these observations. It is well recognized that BAV is a heritable autosomal disease with an incomplete penetrance (*Huntington, Hunter and Chan, 1997; Braverman et al., 2005; Loscalzo et al., 2007*). This reduced penetrance is also observed within many BAV models of mice (*Fernandez et al., 2009; Laforest, Andelfinger and Nemer, 2011; Thomas et al., 2012*). As such is the case with the *Nos3*$^{-/-}$ mouse model in which only ~25% develop BAV whilst 75% of *Nos3*$^{-/-}$, which are genetically identical, develop TAV (*Peterson et al., 2018*). Interestingly, a study by Bosse et al shows that introduction of Notch1$^{+/-}$ haplodeficiency in the *Nos3*$^{-/-}$ background increases the penetrance of BAV to ~60% (7 out of 11) and ~30% developed severe dysplastic valves (3 out of 11) (*Bosse et al., 2013*). Moreover later studies using the Notch1$^{+/-}$;*Nos3*$^{-/-}$ model also showed that combinatorial gene variations could exacerbate BAV related aortopathy (*Koenig et al., 2015*). Our studies show that correct valvular formation requires the careful orchestration of multiple cell lineages and that both BAV and TAV *Nos3*$^{-/-}$ show distinct morphological deviations within the cardiac outflow tract (*Peterson et al., 2018*). Such observations elucidate the complexity of valve formation and suggest that there are likely subtle cell lineage interactions underlying the severity of morphological anomalies within the OFT. As such, the aortic and valvular complications observed at the outflow tract of BAV patients could derive from a spectrum of cell lineage aberrations.

Whilst there is still little understanding regarding any interactions among these early cell lineages, the outcome of morphological effects could substantiate clinical observations from physicians and thoracic surgeons which first attempted to classify the different etymology's of BAV using phenotypes. There are numerous studies using these classification schemes to identify possible differences among patients (*Koenraadt et al., 2016; Li et al., 2019; Lim, Celermajer and Bannon, 2021; Sillesen et al., 2021*). Yet any formal theory explaining how subtypes of BAV could affect disease progression remains ambiguous. There is currently still little agreement

regarding the practicality of using BAV subtypes for patient risk stratification.

Although there are still many questions regarding the origin of BAV, one common goal of many researchers was to understand BAV-related disease progression. From the study of etymologies of BAV came forth the idea that aortic diseases secondary to BAV might result from accumulated hemodynamic stresses due to altered fluid flow of the bicuspid valve. Studies examining the orifice of the BAV recognized that the asymmetric opening created a nonaxial, turbulent transvalvular flow jet (*Robicsek et al., 2004; Girdauskas et al., 2012*). As a consequence of the flow jet it was shown that BAV can introduce local regions of high and low shear stress at the aortic wall (*Robicsek et al., 2004; Girdauskas et al., 2012*). Metrics regarding "cusp opening angle" were introduced by Della Corte and demonstrated prognostic correlations related to aortic growth in BAV patients (*Della Corte et al., 2012*). Moreover BAV was shown to be associated with altered wall shear stress in the ascending aorta substantiating the difference in hemodynamics among TAV and BAV patients (*Barker et al., 2012*). The adoption of 4D flow analysis allowed for accurate evaluation of in vivo fluid dynamics (*Hope et al., 2010*).

Increased hemodynamic mechanical stresses can alter normal VSMCs gene expression patterns and contribute to aortic dilation and dissection in the aortic vessel wall (*Isnard et al., 1989; Robicsek and Thubrikar, 1994; Jensen, Bentzon and Albarrán-juárez, 2021*). A strong argument for the hemodynamic theory of BAV-associated aortopathy is that many BAV patients tent to develop aortic complications during adolescence (*Girdauskas, Borger, et al., 2011*). Which suggests that BAV-related aortopathy is independent from the genetic developmental origin of BAV. Congenital defects are generally thought to give rise to complications during childhood development. Nevertheless, there is agreement among researchers that genetic predisposition can influence susceptibility to disease. Studies examining Loeys-Dietz syndrome or Marfan have shown that patient offspring carry increased risk to develop complications at later stages in life (*Mariko et al., 2011; MacCarrick et al., 2014*). However much is still unclear regarding BAV associated aortopathy, although there is plenty of evidence to suggest that anomalous aortic valve development is intrinsically linked to development of multiple cardiac structures such as the aorta, coronary arteries and aortopulmonary complex (*Henderson, Eley and Chaudhry, 2020*). Moreover, patients of first degree relatives of BAV often show increased susceptibility for aortic complications independent of having BAV (*Biner et al., 2009*). Cases of "young adults" developing aneurysms secondary to BAV do suggest that hemodynamics is not the only factor influencing disease progression (*Burks et al., 1998; Tzemos et al., 2008; Michelena et al., 2011*). Our studies (chapter 5) show cases of *Nos3*$^{-/-}$ mice developing aneurysms and a case of

dissection after 4 months which is similar to *Robo4* deficient BAV mice that develop aneurysms at 20 weeks (*Gould et al., 2018; Peterson et al., 2020*). These observations elucidate that there is currently still a gap in understanding regarding temporal related factors that change during aging but have an implicit role for to the formation of aortic aneurysms.

7.2 Future Perspectives

The current general consensus among cardiovascular researchers is that BAV related aortopathy is a result caused by a combination of both genetic predisposition and hemodynamic adaptation. This explanation, whilst arguably true, holds little value if the impact of genetic predisposition and the magnitude of hemodynamic adaptation cannot be accurately quantified within the same system. Knowing that BAV related aortopathy develops considerably later in life than BAV itself means that secondary effects should take hold before disease onset. A possible approach would be study cellular markers of vascular adaptation to complement current genetic investigations. This could lead to new insight into the temporal relation between early genetic predisposition and later hemodynamic adaptation. Ideally these studies would address the challenge of which mechanisms genetic predisposition affects to increase disease susceptibility and how such effects transition into phenotypical hemodynamic adaptation. One option could be to investigate epigenetic signatures related to vascular aging within the context of BAV related aortopathy. Numerous studies have already been using methylation status of genes such as PTEN (*Ma et al., 2018*), PDGF (*Zhang et al., 2012*), MYH11 (*R. Liu et al., 2013*) as indicators of vascular aging in vascular smooth muscle cells (*Xu, Li and Liu, 2021*). Given that our *Nos3*$^{-/-}$ mouse model develops both BAV and BAV-related aortopathy as a result of anomalous neural crest contributions, a future direction could investigate both the genetic and epigenetic components of derivative cells during disease progression within the same system. The combination of a cellular methylation mark and a RNA expression profile could reveal insight into the molecular intricacies underlying the phenotypical alterations in BAV disease because age related changes in DNA methylation are known to affect gene expression (*Razin and Cedar, 1991; Salameh, Bejaoui and El Hajj, 2020*).

Combinatorial genetic and epigenetic profiling would allow for more detailed characterization of the neural crest derived populations than gene expression levels alone. Moreover, this approach could possibly identify cellular signatures characteristic of vascular integrity and disease progression within these cell populations because the combination of an methylation mark and a RNA expression profile could reveal insight into the molecular intricacies underlying the phenotypical alterations in BAV disease. DNA methylation profiling has also been shown

to be successfully applied as biomarker in distinguishing cancer types, such as breast, lung, prostate, and colorectal cancers using blood based cell free liquid biopsies (*Constâncio et al., 2020*). The discovery of novel markers and proxies can facilitate the translation of disease status in BAV patients. Such markers would allow for quantitative measurements to more accurately evaluate the patient risk status, and thus improve clinical decision-making regarding adequate intervention strategies. Future research should therefore focus to resolve the implications and contributions of both genetic and epigenetic influences on the development of BAV and BAV-related aortopathy.