Developmental cell lineage dynamics in Bicuspid Aortic Valve disease

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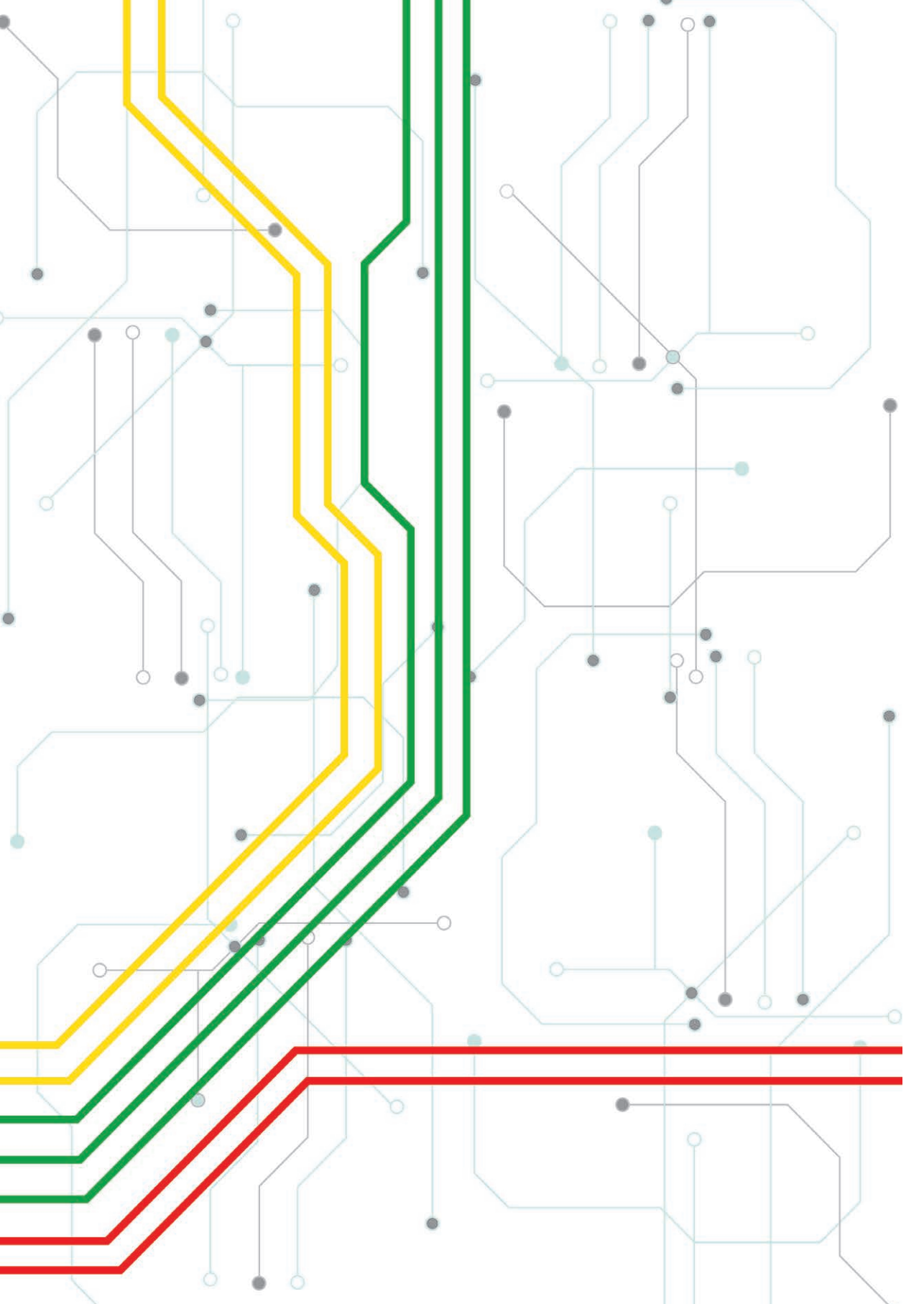

Summary/Samenvatting

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The bicuspid aortic valve (BAV) is a congenital heart defect which is characterized by the formation of two aortic leaflets instead of the normal three leaflets within the tricuspid aortic valve (TAV). Whilst all BAV patients have a bicuspid valve, extended patient monitoring has revealed a large variation of disease progression trajectories during a patient's lifetime. This large variation troubles clinical decision making due to the uncertain proliferation of BAV disease. More knowledge of the biological mechanisms underlying BAV could address that uncertainty and thus help stratify patient risk with more accuracy. Therefore this thesis aims to advance our current understanding regarding the biological impact and developmental mechanisms underlying congenital BAV and BAV related aortopathy.

In **Chapter 1** information is presented on several aspects of cardiovascular development that are relevant to study the developmental anomalies relating to BAV. Background information regarding the consequences of BAV, aortopathy and the role of cell lineages involved in outflow tract formation is provided along with an outline of the chapters within this thesis.

Chapter 2 focusses on the role of cell tracing and fate mapping experiments in cardiac outflow tract (OFT) development. Common among many congenital heart diseases affecting the OFT, such as BAV, is a large variation in disease phenotypes. Embryonic fate mapping and lineage tracing experiments have categorized and studied many of the individual cell lineages involved in OFT formation. Nevertheless it remains challenging to relate cell lineage dynamics to the morphologic variation observed in OFT pathologies. In this chapter we provide an overview of historical fate mapping and cell tracing techniques used to study OFT development and introduce emerging technologies which will provide new opportunities to aid our understanding of the cellular dynamics underlying OFT pathology.

Chapter 3 addresses a common methodological concern involving cellular measurements. Many biological studies highly value the quantification of specific cells within a localized tissue sample or an in-vitro cell culture because this provides quantitative information regarding cell behavior under various circumstances. Numerous methods to address cell quantification have thus been developed to address this issue, ranging from manual cell counting to automated image analysis tools. ImageJ is a popular tool that enables researchers to create custom image analysis scripts to facilitate automated image analysis. Whilst there exist many tutorials on the internet to quickly develop such custom analysis methods, there is often surprisingly little

concern regarding any aspects of measurement validation and process-data collection to ensure experimental reproducibility. Therefore we have developed a software script specific to ImageJ to facilitate collection of process-data for measurement assurance. This script simultaneously aids researchers with exercising good research practices when performing computational image analysis.

In **Chapter 4** we investigate the development of BAV using the *Nos3*$^{-/-}$ mouse model. We aimed to understand whether alterations in early cell lineages contributed to the formation of BAV and influenced cardiovascular outflow tract development. To understand the role of early cell lineages during OFT formation lineage tracing experiments were performed to evaluate cell lineage contributions to the heart over multiple developmental stages. A detailed overview was constructed describing a novel interpretation of aortic and pulmonary valve development using high resolution microscopic imaging and 3D reconstruction. Using this approach it was determined that *Nos3*$^{-/-}$ mice develop a BAV without a raphe as a result of incomplete separation of the parietal outflow tract cushion into the right and non-coronary leaflet. Further investigation into the formation of aortic and pulmonary leaflets showed that the individual leaflets harbour unique cell lineage contributions. The right and left leaflets of the aortic valve contains relatively more neural crest derived cells, whereas the non-coronary leaflets contains relatively little neural crest derived cells and more second heart field derived cells. Careful inspection of these lineage populations in *Nos3*$^{-/-}$ revealed altered deposition of neural crest cells and second heart field cells within the parietal outflow tract cushion of *Nos3*$^{-/-}$ embryos. The effects of these abnormal cell lineage distributions also affected the positioning of the aortic and pulmonary valves at the orifice level. Our results demonstrated a small deviation in the distribution of neural crest and second heart field populations affected normal valve formation and resulted in the characteristic right-non type BAV in *Nos3*$^{-/-}$ mice.

Chapter 5 investigates the effects of *Nos3*$^{-/-}$ on vascular health. Given that patients with BAV are at increased risk of aortopathy this chapter examines BAV-associated aortopathy in *Nos3*$^{-/-}$ mice. Here we used a combination of histological examination and in-vivo ultrasound imaging as an initial step to investigate aortic dilation and dissections in *Nos3*$^{-/-}$ mice. We discovered spontaneous aortic dissections in ascending aortas located at the sinotubular junction in ~13% of *Nos3*$^{-/-}$ mice. Further analysis showed that *Nos3*$^{-/-}$ mice were prone to develop aortic dilations in the proximal- and distal-ascending aorta during early adulthood. To explain these observations we examined the elastic fiber and collagen content within the aortic wall using classical histological stainings. Analysis of the elastic fibers determined that the aortic walls

of *Nos3*$^{-/-}$ contained less elastin than those of wild type mice. Interesting is that the vascular smooth muscle cells (VSMCs) which make up the aortic vessel wall are derived from neural crest and second heart field lineages during development. Investigation of the neural crest lineage in the ascending aorta showed a reduction of neural crest derived VSCMs at the inner media of the aortic wall that could be traced back to embryonic development. Using single cell RNA sequencing we compared the gene expression profiles of VSMCs between wild type mice and *Nos3*$^{-/-}$. We found downregulation of 15 genes of which 7 were associated with aortic aneurysms and dissections in the human population. Elastin mRNA was most markedly downregulated, followed by Fibulin-5 expression, both primary components of elastic fibres. This chapter demonstrates that disrupted endothelial mediated NO signalling in mice can give rise to aortic dilation and dissection as a consequence of inhibited elastic fibre formation in VSMCs within the ascending aorta of *Nos3*$^{-/-}$mice.

In **Chapter 6** we address the pathological formation of pulmonary ductal coarctation (PDC) and left pulmonary artery interruption during development. To study this formation a combined approach was adopted using both mouse embryos and stage-matched human embryos. Neural crest and second heart field cell lineages were studied in perspective of the VEGF120/120 mutant mouse strain that develops pulmonary atresia. Careful observations showed that pulmonary stenosis/atresia and a subsequent lack of proper incorporation of the ventral segment into the aortic sac were the result of an anomalous development related the asymmetric contribution of second heart field to the future pulmonary trunk on the left side of the aortic sac, known as, the pulmonary push. These findings explain how neural crest-derived muscular ductus arteriosus (DA) tissue may continue contraction and stenosis formation even after birth leading to the postnatal development of proximal left pulmonary artery interruption. Surgical intervention strategies of PDC should aim to eliminate DA tissue instead of stenting this segment, as this will lead to less complication on the whole.

Chapter 7 contains a general discussion addressing some of the current challenges in BAV research within the context of the chapters presented in this thesis. Here we discuss the consequence of developmental predisposition theories relative to findings from different research studies. We suggest future directions of research that could address current study limitations to further expand our understanding of the intricacies of cardiovascular development and BAV.