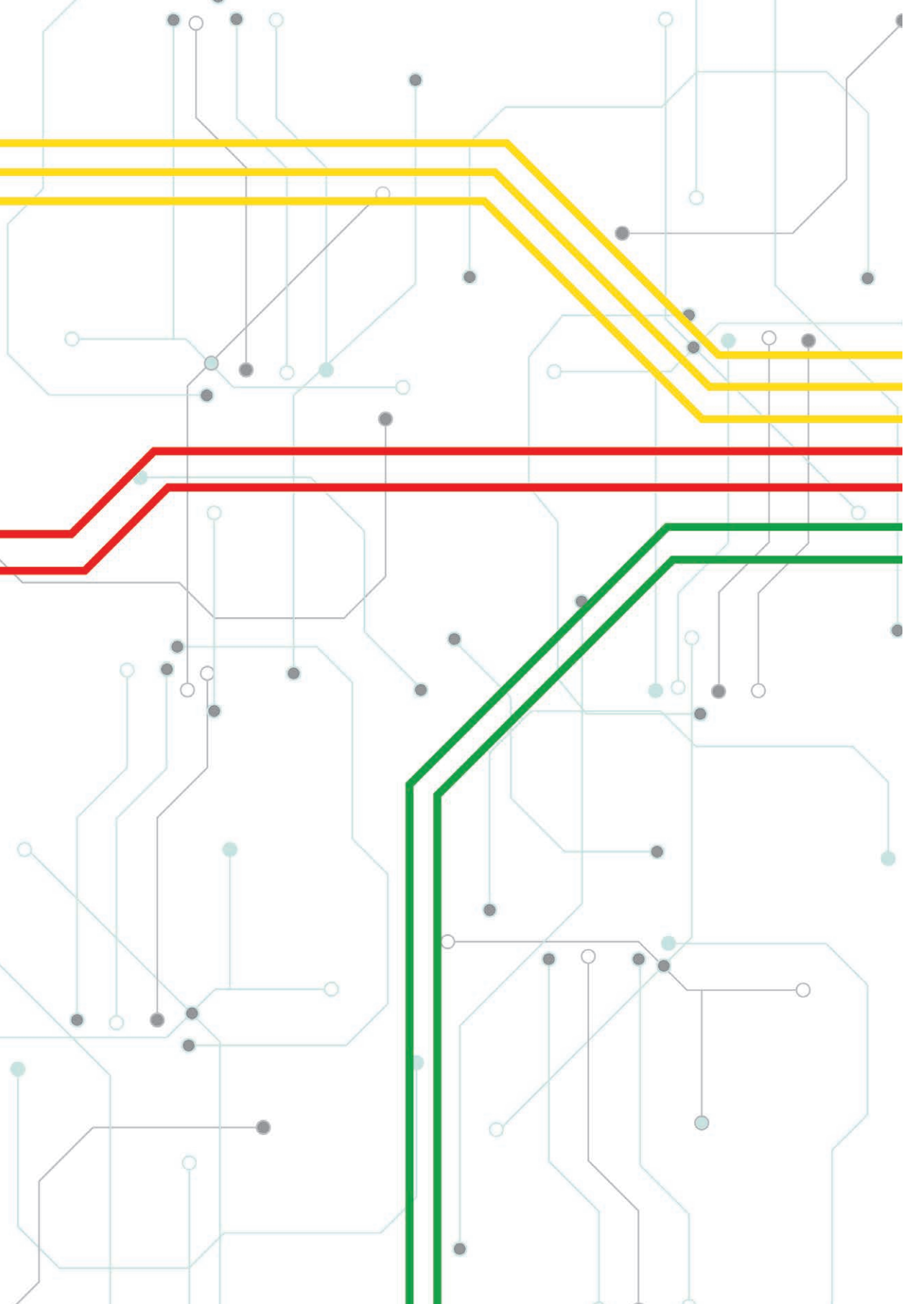
Developmental cell lineage dynamics in Bicuspid Aortic Valve disease

Peterson, J.C.



Chapter 1

Introduction

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1.1 Background

Bicuspid aortic valve (BAV) is a congenital heart defect in which the aortic valve contains only two instead of three cusps. BAV is the most common congenital heart defect, estimated to affect 1-2% of the general population (*Hoffman and Kaplan, 2002*) and occurs three to four times more frequent in males than females (*Kong et al., 2020*). BAV can have multiple configurations depending on the fusion of the aortic leaflets (**Fig. 1**). As such, BAV can be classified into three categories; fusion of the right and left coronary cusps (RC/LC) (**Fig. 1C**) which occurs in ~60% of patients, fusion of the right and non-coronary cusps (RC/NC) (**Fig. 1D**) occurring in ~30% of patients, and finally fusion of the left and non-coronary cusp (LC/NC) (**Fig. 1E**) occurring in ~4% of patients (*Fernandes et al., 2004; Sievers and Schmidtke, 2007; Sun et al., 2018*). Patients with BAV have an increased risk of aortic valve stenosis, which is a result of the narrowing of the outflow tract due to insufficient opening of the aortic cusps (*Aydin et al., 2013; Verma and Siu, 2014*). Moreover, inadequate functioning of the aortic valves can also lead to aortic regurgitation (*Braverman et al., 2005*). Another complication, is the increased risk of developing a dilation in the aortic vessel known as an aortic aneurysm which can lead to aortic rupture or dissection (*Fedak et al., 2002*). Aortic aneurysms can develop in the thorax or the abdominal part of the aorta. Within this thesis we use the terms thoracic aortic aneurysm and aortic aneurysms interchangeably because within the context of BAV, patients are more prone to develop thoracic aortic aneurysms over abdominal aortic aneurysms than the general population (*Ward, 2000; Shim et al., 2011; Aydin et al., 2013; van de Pol et al., 2017*).

Whilst aortic valve stenosis and aortic regurgitation are often considered a direct result of the aberrations in aortic valve morphology, the mechanisms through which BAV affect the integrity of the aortic wall in aortic aneurysms and dissection are less well understood. Such gaps in knowledge affect clinical decision making and have direct consequences for patient management and intervention strategies. Given the unpredictable lifetime risk of acute aortic emergencies related to aortic wall pathology in BAV, there has been much debate regarding common guidelines for surgical intervention (*Boodhwani et al., 2014; Michelena et al., 2014*). Historically, aortopathy observed in BAV patients was treated identical to aortopathy in patients with a normal tricuspid aortic valve (TAV). However, as more research supported a strong genetic component underlying BAV-associated aortopathy, the risk of acute aortic complications was estimated substantially higher in BAV (*Fedak et al., 2005; Biner et al., 2009*). Such insights led to recommendations for a more aggressive surgical approach similar to guidelines for patients with Marfan syndrome (*Hiratzka et al., 2010*). More recent studies and

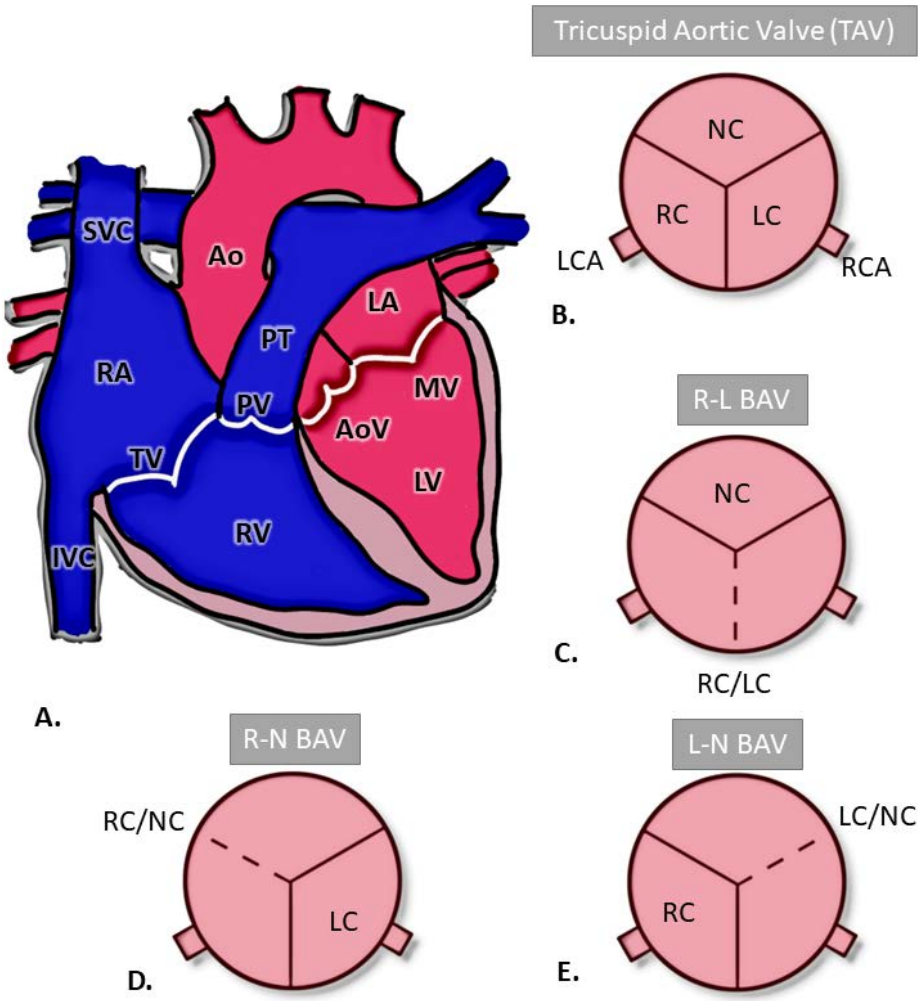

Figure 1.1 Introduction to BAV. A: *A schematic overview of the human heart. Oxygen deprived blood enters the heart on the right-side (blue). After the blood passes the pulmonary circulation it returns to the heart as richly oxygenated blood from where it continues into the systemic circulation (red).* **B**: *The tricuspid aortic valve contains three aortic leaflets, the left coronary cusps (LC) is adjacent to the inlet of the left coronary artery (LCA). The right coronary cusp (RC) is positioned next to the inlet of the right coronary artery (RCA). The non-coronary cusp (NC) is third leaflet and is not situated next to any of the coronary inlets.* **C**: *Bicuspid aortic valve (BAV) subtypes can be distinguished on the location of leaflet fusion. R-L type BAV have fused leaflets of the RC and LC.* **D**: *A R-N type BAV is observed when the RC is connected to the NC.* **E**: *A L-N type BAV can be determined if the LC and the NC are fused. Abbreviations: Ao: Aorta, AoV: Aortic Valve, LA: Left Atrium, LCA: Left Coronary Artery, LC: Left Coronary Cusp, LV: Left Ventricle, MV: Mitral Valve, NC: Non-Coronary Cusps, PV: Pulmonary Valve, RA: Right Atrium, RCA: Right Coronary Artery, RC: Right Coronary Cusps, RV: Right Ventricle, PT: Pulmonary Trunk, TV: Tricuspid Valve, SVC: Superior Vena Cava, IVC: Inferior Vena Cava.*

observations have led to a middle ground approach suggesting that BAV aortopathy is less dangerous than previously described, but still requires careful patient monitoring (*Itagaki et al., 2015; Sherrah et al., 2016; Borger et al., 2018; Otto et al., 2021*). Determining the underlying cause of BAV associated aortopathy is thus vital for patient risk stratification and operative management. To achieve this, a detailed understanding of the cellular mechanisms influencing the embryological development of BAV and the aorta is required.

1.2 Anatomy of the Human Heart

The heart is a muscular organ which main function is to pump blood with nutrients and oxygen throughout the body (*Silverthorn, 2009*). It consists of four chambers, four valves, coronary arteries, and the cardiac conduction system. The hearts functionality can be separated in a right-sided pulmonary circulation and a left-sided systemic circulation (**Fig. 1.1A**). The gatekeepers of these two circulatory systems are known as the semilunar valves which derive their name from their resemblance to a crescent moon. The human heart contains two semilunar valves which are embedded in the cardiac outflow tract. One is the pulmonary valve which connects the heart to the pulmonary trunk. The other is the aortic valve which connects the heart to the aorta. The semilunar valves have the important function of maintaining unidirectional blood flow throughout the body. In healthy adults this function is regulated by the synchronized opening and closing of the three cusps (tricuspid) within each valve. When the heart contracts (systole), the ejecting blood from the chambers will drive the cusps into an open position allowing blood to flow into the connected arteries. When the heart dilates (diastole), the sudden change in pressure, enables the valves to close preventing blood in the arteries to leak back into the chambers. During a single heart beat the heart powers both the pulmonary and systemic circulation. The pulmonary circulation starts as deoxygenated blood enters the heart through the caval veins into the right atrium after which it flows into the right ventricle passing the tricuspid atrioventricular (AV) valve. Thereafter, the blood passes the tricuspid leaflets of the pulmonary valve and continues its route into the pulmonary trunk which transports the blood into the lungs via multiple pulmonary arteries. Once the deoxygenated blood passes the pulmonary circuit and the gasses (CO_2/O_2) have been exchanged, it returns back to the heart as richly oxygenated blood. This oxygen rich blood enters the left atrium via the pulmonary veins. After which it continues into the left ventricle through the mitral AV valve. Upon contraction of the left ventricle the blood exerts a hydrodynamic force large enough to open the aortic semilunar valve, enters the aorta, and continues its journey too supply oxygen to every tissue in the body. The heart is the first to receive oxygenated blood

via the coronary arteries. Given that the aorta is the main artery of the human body, changes affecting the vessel wall integrity can have a major influence on homeostasis. A common aortic adaptation which happens during adulthood is the loss of aortic elasticity due to the stiffening of the elastic lamella within the aortic wall. Stiffening of the aortic wall will generally result in increased blood pressure which also increases cardiovascular risk (*Benetos et al., 2010; Safar et al., 2018*). Studies show that increased systemic blood pressure strongly correlates with aneurysm formation (*Kobeissi et al., 2019*) and has been linked to genetic heritability (*Biddinger et al., 1997; Albornoz et al., 2006*).

1.3 Cardiac embryology

1.3.1 Formation of the four-chambered heart

Cardiovascular development is a complex but organized process that is highly depended on the correct proliferation, migration and differentiation of various cell types of multiple embryonic origins.

The heart originates from the anterior splanchnic mesoderm of the embryonic plate (**Fig. 1.2A**). During gastrulation, these mesodermal cells arise from the primitive streak and subsequently migrate cranially and laterally to form the bilateral cardiogenic plates (primary heart fields) which will give rise to the myocardial and endothelial cell lineages (*DeRuiter et al., 1992; Lough and Sugi, 2000*). The expression of NKX2.5 (*Olson and Srivastava, 1996*) and GATA4 (*Laverriere et al., 1994*) within the cardiogenic plates induces the differentiation of early cardiomyocytes. The fusion of the two cardiogenic plates results in the formation of the muscular primary heart tube containing an inner endothelial and outer myocardial layer (*DeRuiter et al., 1992*). After formation of the primary heart tube, the embryonic heart will perform a number of dynamic changes through a complex looping process. During the looping process the heart tube elongates by addition of cardiomyocytes at both poles of the tube and bends into a s-shape heart tube (*Kelly and Buckingham, 2002*) (**Fig. 1.2B**). The curves of the s-shaped heart tube form the primitive atrial and ventricular chambers (*Moorman and Christoffels, 2003*). These primitive chambers are connected by the atrio-ventricular canal (AVC). The regions of the AVC and the outflow tract (OFT) will develop local tissue swellings within the endocardium, a process known as cushion formation. These regionalized cushions will later develop into the atrioventricular (mitral and tricuspid) and semilunar (aortic and pulmonary) valves. The formation of a four-chambered heart is final when septation of the atria, AVC, ventricles and outflow tract is complete.

1.3.2 Semilunar valve development

As the s-shaped heart tube takes shape a thick basement membrane is formed between the endothelial and myocardial layer at the regions of the OFT, aptly called "cardiac jelly". The cardiac jelly is a hydrophilic substance rich in hyaluronic acid, proteoglycans and extracellular matrix secreted by the myocardium (*Eisenberg and Markwald, 1995*). Moreover, the myocardial secretion of factors of the TGFβ super family, such as BMP2 and TGFβ-2, induces an endothelial to mesenchymal transition (EndMT) within the endothelial cells lining the heart tube (*Ma et al., 2005; Kruithof et al., 2012*). The process of EndMT results in a morphologic transition of static coble-stone endothelial cells into a mobile spindle mesenchymal cellular phenotype. The mesenchymal cells then migrate into the cardiac jelly populating the OFT cushions. Cells from other lineages such as the second heart field and neural crest cells will also migrate into the cushions resulting in further remodelling forming the septal and parietal cushions of the OFT (**Fig. 1.2C**). During normal development the septal and parietal cushions will fuse forming part of the aortopulmonary septum separating the aorta from the pulmonary trunk (**Fig. 1.2D**) and the formation of the aortic and pulmonary valve, each containing 3 leaflets. As the leaflets mature into cusps, three distinct layers become distinguishable within each leaflet, the collagen-rich fibrosa located at the apical (arterial) side of the valve, a spongiosa middle layer composed of fibroblasts, mesenchymal cells, and a mucopolysaccharide-rich matrix, and located at the basal (ventricular) side of the valve is the ventricularis which is high in elastin. These layers provide tensile strength and flexibility to the valves (*Freeman and Otto, 2005*).

1.3.3 Cell lineages contribution to the cardiovascular OFT

The First and Second Heart Field

The first heart field (FHF) is an embryonic region located near the cardiac crescent (*Zaffran and Kelly, 2012; Später et al., 2013*). The cells of the FHF form the earliest cardiac progenitor cell population to be observed during development. The cells of the second heart field (SHF) are the second wave of cardiac progenitor cells to form (*Waldo et al., 2001; Kelly, Brown and Buckingham, 2001; Mjaatvedt et al., 2001*). The SHF progenitors are located posteriorly to the heart tube in the pharyngeal mesoderm. The FHF contributes to the formation of the myocardium of the left ventricle and atrial appendages, with a minor contribution to the right ventricle whilst the second lineage gives rise to the myocardium of the OFT, right ventricle, and the posterior wall of the atria, corresponding to SHF-derived parts of the heart (*Meilhac et al., 2004*).

During development, the SHF lineage is multipotent and does not only provide cardiomyocytes

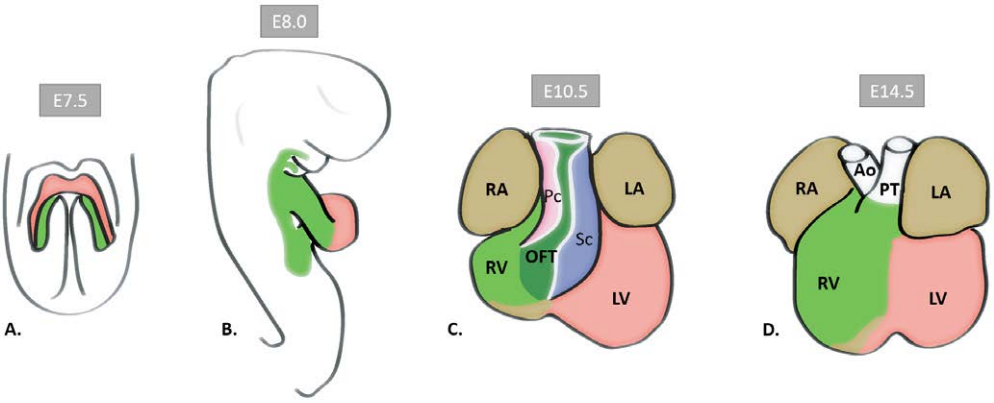

Figure 1.2 Murine Embryonic Heart development. A: *During heart development early cardiac progenitor populations can be distinguished as cells of the first heart field (red) and second heart field (green).* **B**: *During the formation of the heart tube the FHF contribute to the formation of the future left ventricle. The SHF cells are added to both poles of the elongating heart tube. As a result the SHF will contribute to the development of the cardiac atria, the right ventricle and the outflow tract (OFT).* **C**: *At E10.5 the left ventricle is primarily derived from cells of the FHF whilst the right ventricle is mostly derived of SHF cells. At this stage the aorta and pulmonary trunk still share a common outflow tract and two outflow tract cushions, the parietal (pink) and septal cushions (blue) will accommodate cells of endothelial, neural crest and SHF lineages during outflow tract septation.* **D**: *At E14.5 the process of outflow tract septation is completed. Abbreviations: Ao: Aorta, LA: Left Atrium, LV: Left Ventricle, OFT: Outflow Tract, Pc: Parietal Cushion, Sc: Septal Cushion, PT: Pulmonary Trunk, RA: Right Atrium, RV: Right Ventricle.*

(*Waldo et al., 2001; Kelly, Brown and Buckingham, 2001; Zaffran et al., 2004*) but also contributes to the smooth muscle cells located at the base of the aorta and pulmonary trunk (*Waldo et al., 2005; Sun et al., 2007; Sawada et al., 2017*) and the valvular interstitial cells within the aortic and pulmonary valves (*Eley et al., 2018; Lioux et al., 2020*).

The endothelial cells

A crucial step for valve development is the formation of endocardial cushions. Studies examining OFT formation describe the formation of regional swellings filled with cardiac jelly between the myocardial wall and the endothelial lining (*Mjaatvedt and Markwald, 1989; Eisenberg and Markwald, 1995; Schroeder et al., 2003*). Endothelial cells lining the cardiac cushions will undergo and populate the aortic cushions. These endothelial derived mesenchymal cells form the bulk of the valvular tissue within the semilunar valve leaflets (*Eisenberg and Markwald, 1995; Sugishita, Watanabe and Fisher, 2004*).

The cardiac neural crest cells

The neural crest is a population of multipotent progenitors originating from the neural fold in the region of the otic placode and the posterior border of somite 3 (*Keyte and Hutson, 2012; Keyte, Alonzo-Johnsen and Hutson, 2014*). Early lineage tracing studies using chick-quail models were the first to notice the contribution of the neural crest lineage to the cardiovascular system (*Kirby, Gale and Stewart, 1983; Le Douarin, 2004*). During development the neural crest cells (NCCs) migrate from the neural fold over the arterial arches into to the arterial pole of the heart and induce outflow tract remodelling. The NCCs contribute to the aortopulmonary valves, arterial wall, cardiac ganglia and the aortopulmonary (AP) septum (*Bergwerff et al., 1998; Poelmann, Mikawa and Gittenberger-De Groot, 1998; Waldo et al., 1998*). The AP septum divides the embryonic common arterial trunk into the pulmonary artery and aorta (*Waldo et al., 1998, 1999*). Later lineage tracing studies using the Cre-LoxP system to genetically label the NCCs and its derivatives further substantiated the intricate role of NCCs in mammalian heart development (*Jiang et al., 2000*).

1.4 Aortopathy and BAV: a common origin

BAV was historically interpreted as an anomalous congenital variation limited to effect valvular morphology (*Pomerance, 1972; Boudoulas, 2003*). Current studies suggested BAV to be a more complex disorder of which the effects are not limited to valvulogenesis (*Ward, 2000*). Evidence suggests that BAV disease could also directly and/or indirectly affect the integrity of the aorta and connected cardiac structures (*Loscalzo et al., 2007; Rajan Jain et al., 2011; Grewal et al., 2014*). BAV patients were shown to have, throughout their lifetime, an increased risk of developing serious complications, including sudden cardiac death, severe aortic valve dysfunction, endocarditis, aortic aneurysm or dissection and left ventricular dysfunction (*Siu and Silversides, 2010; Michelena et al., 2011; Laforest and Nemer, 2012; Mordi and Tzemos, 2012*). Most notable in BAV patients are the nonvalvular complications that occur in up to 50% of adults with BAV of which the most common abnormality is a dilation of the aortic root and the thoracic aorta (*Siu and Silversides, 2010; Michelena et al., 2011; Mordi and Tzemos, 2012*).

Based on pedigree analysis and familial clustering of BAV patients, researchers suspected a genetic basis for the development of BAV. These early genetic linkage studies found increased prevalence of BAV in patient family members compared with the general population supporting a heritable component of the disease (*Emanuel et al., 1978; Huntington, Hunter*

and Chan, 1997; Cripe et al., 2004). With the advances in DNA sequencing technologies many gene mutations have been found causal to BAV. Development of knockout murine models allowed for the study of familial BAV. Humans and mice with genetic defects in the TGF-β/BMP signalling pathway such as *Smad6* (*Gillis et al., 2017*) and *Alk2* (*Thomas et al., 2012*) affect the endothelial cell lineage through defects in EndMT resulting in BAV. Patients with defects in genes related to cardiac progenitors such as *Nkx2.5* (*Qu et al., 2014*), *Gata4* (*Li et al., 2018*) *Gata6* (*Xu et al., 2018*) are also known to develop BAV. Similar to these patients, mice with defects in SHF derived lineages such as *Gata5* (*Laforest, Andelfinger and Nemer, 2011*) and *Gata6* (*Xu et al., 2018*) also develop BAV. Moreover, genetic alterations affecting NCC lineages such as *Krox20* (*Odelin et al., 2017*), *Pax3* (*Rajan Jain et al., 2011*), or *Hoxb1* (*Zaffran et al., 2018*) have also been shown to give rise to BAV in mice. Genetic defects affecting these early cardiac lineages may not only affect formation of the aortic valve but also affect development of other cardiac components derived from these same cell lineages (*Grewal et al., 2014*).

BAV and associated thoracic aortic aneurysms are thought to be manifestations of a common genetic defect. This is supported by observations that BAV patients tend to have a more progressive dilation of the ascending aorta than TAV patients, even after aortic valve replacement (*Russo et al., 2002; Yasuda et al., 2003*). Moreover, children and young adults with BAV seem predisposed to develop aortic complications as these have larger aortic dimensions of the root and the ascending aorta in conjunction with reduced elastic properties of the aortic vessel wall when compared to children with TAV (*Nistri et al., 1999; Basso et al., 2004; Oulego-Erroz et al., 2013; Blais et al., 2020*). First degree relatives of BAV patients (with normal functioning TAVs) have an increased likelihood of developing aortic dilations as well as abnormal elastic properties in the aortic wall (*Biner et al., 2009*). BAV disease is also known to coexist with other congenital vascular defects with cells of common developmental origin; such as coarctation of the aorta. Of all patients diagnosed with coarctation of the aorta, approximately 50% to 75% also have BAV (*Sinning et al., 2018*). Moreover, BAV is also associated with and genetically related to hypoplastic heart syndrome. There are a number of syndromes whose cardiac involvement includes BAV such as Williams syndrome with supravalvular stenosis, Shone's syndrome with inflow and outflow obstructions, Jacobsen syndrome with various left sided heart lesions and Turner syndrome with coarctation of the aorta and aortic arch abnormalities. Other cardiac malformations that have been associated with BAV include ventricular septal defects (*Duran et al., 1995*), patent ductus arteriosus (*Gelb et al., 1999; Quintero-Rivera et al., 2015*), and atrial septal defects (*Hor et al., 2008*) suggesting extensive genetically induced cell lineage aberrations originating during cardiac development as a basis for BAV related complications.

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1.5 Thesis Outline

The aim of the research described in this thesis is to advance our current understanding of the impact and mechanisms underlying congenital BAV and BAV related aortopathy. We anticipate that better understanding of congenital BAV could explain the increased susceptibility of aortopathy in patients with BAV and could contribute to the identification of novel parameters to more accurately determine patient risk stratification.

In **Chapter 2** we provide an extended overview of general recognized lineage tracing methodology. Given the large implications of lineage tracing experiments to our current understanding of heart development, this chapter aims to elucidate the technical advances and limitations of cell lineage tracing methods with respect to their roles in cardiac outflow tract development. Here we will address current challenges and discuss emerging opportunities for this field of study.

To effectively study cell lineage contributions, a method was required to examine cellular contribution. In **Chapter 3** we demonstrate an analysis method based on fluorescent markers within the boundaries of the tissue of interest. During our studies we developed a method for efficient analysis of cellular contribution using an image analysis pipeline specific to immunofluorescent stained tissues sections.

BAV has been described to result from defects in cushion tissue formation within the outflow tract during development. In **Chapter 4** we explore the formation of BAV in our *Nos3* mutant model and study the contribution of endothelial, neural crest, and second heart field cell lineages to the aortic valve throughout embryonic development. We aimed to identify congenital cell lineage aberrations to that could elucidate the critical processes required for proper valve formation.

In **Chapter 5** we dive deeper in the phenotypical implications of the anomalous cell lineage contributions to the cardiac outflow tract. Observations in BAV patients suggest the possibility of genetic predisposition for BAV related aortopathy. This study aims to examine the role of *Nos3* deficiency on the cell lineages that contribute to the formation of the aortic vessel wall. The dynamics of the neural crest cells and second heart field derived smooth muscle cells is important to cardiac development and formation of the great arteries.

In **Chapter 6** we examine the role of neural crest and second heart field lineages in the formation of pulmonary ductal coarctation and left pulmonary artery interruption.

Chapter 7 contains a general discussion addressing the current challenges in BAV research within the context of the chapters presented in this thesis, and an outlook on future perspectives.