Aortic Valve Stenosis - an understanding of the disease process from fetus to adults and, an insight into its treatment.

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Thesis submitted for the degree of

Doctor of Philosophy
Declaration of Work

I, Saadullah Husayn Ahmed, confirm that the work presented in this thesis is my own, carried out under the supervision of Prof Cook and Prof Lovering. Where information has been derived from other sources, I confirm that this has been indicated in the text.

Signature: ............................................

Date:
For

Mama and Baba
Acknowledgements

I would firstly like to offer my gratitude to The Almighty; without His Love, Grace, Guidance and Strength, I would have found myself lost - all praise belongs to Him. I would then like to thank my supervisors, Professor Andrew Cook and Professor Ruth Lovering – they helped design a path when I felt I had nowhere to go, and have since then been there to encourage me, wipe off my tears and remind me to keep going until I get it done. From getting me to understand the basics in the fields of Cardiac morphology and Gene Ontology, to the long hours going through my work, to the many revisions of this thesis, they helped me shape and develop my research. This PhD would not have been possible without their continuous support, help and guidance. I will forever be grateful.

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<th>Description</th>
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<tbody>
<tr>
<td>AR</td>
<td>Aortic Regurgitation</td>
</tr>
<tr>
<td>AS</td>
<td>Aortic Stenosis</td>
</tr>
<tr>
<td>AV</td>
<td>Atrio-Ventricular</td>
</tr>
<tr>
<td>AVC</td>
<td>Atrio-Ventricular Canal</td>
</tr>
<tr>
<td>AVD</td>
<td>Aortic Valve Disease</td>
</tr>
<tr>
<td>AVIC</td>
<td>Aortic Valvar Interstitial Cells</td>
</tr>
<tr>
<td>AVR</td>
<td>Aortic Valve Replacement</td>
</tr>
<tr>
<td>B4GALNT2</td>
<td>β-1,4-N-acetylgalactosaminyl transferase-2</td>
</tr>
<tr>
<td>BAV</td>
<td>Bileaflet or 'Bicuspid' Aortic Valve</td>
</tr>
<tr>
<td>BHV</td>
<td>Biological Heart Valve</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAVD</td>
<td>Calcific Aortic Valve Disease</td>
</tr>
<tr>
<td>CHD</td>
<td>Congenital Heart Disease</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CMAH</td>
<td>Cytidine Monophospho-N-Acetylneuraminic acid Hydroxylase</td>
</tr>
<tr>
<td>CMR</td>
<td>Cardiac Magnetic Resonance imaging</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross-Sectional Area</td>
</tr>
<tr>
<td>CSAA</td>
<td>Cross-Sectional Area of Aorta</td>
</tr>
<tr>
<td>CSNK1A1</td>
<td>Casein kinase 1 alpha 1</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CTNBB1</td>
<td>Beta-Catenin</td>
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<tr>
<td>DAAM1</td>
<td>Dishevelled Associated Activator of Morphogenesis 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DAG</td>
<td>Directed Acylic Graph</td>
</tr>
<tr>
<td>DBA</td>
<td>Dolichos Biflorus Agglutinin</td>
</tr>
<tr>
<td>DSH</td>
<td>Disheveled</td>
</tr>
<tr>
<td>DVL</td>
<td>Disheveled (mammalian)</td>
</tr>
<tr>
<td>DXR</td>
<td>Delayed Xenograft Rejection</td>
</tr>
<tr>
<td>EBI</td>
<td>European Bioinformatics Institute</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-Cellular Matrix</td>
</tr>
<tr>
<td>ECV</td>
<td>Extra-Cellular Volume</td>
</tr>
<tr>
<td>EFE</td>
<td>Endocardial FibroElastosis</td>
</tr>
<tr>
<td>eHLH</td>
<td>evolving Hypoplastic Left Heart</td>
</tr>
<tr>
<td>eHRH</td>
<td>evolving Hypoplastic Right Heart</td>
</tr>
<tr>
<td>EMT</td>
<td>Endothelial-to-Mesenchymal Transition</td>
</tr>
<tr>
<td>FAV</td>
<td>Fetal Aortic Valvuloplasty</td>
</tr>
<tr>
<td>FCI</td>
<td>Fetal Cardiac Interventions</td>
</tr>
<tr>
<td>FP</td>
<td>Fetal and Pediatric</td>
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<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose-α-1,3-galactose</td>
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<tr>
<td>GGTA1</td>
<td>Glycoprotein Alpha-GalactosylTransferase 1</td>
</tr>
<tr>
<td>GMP</td>
<td>Gottingen Mini-Pig</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>GTKO</td>
<td>Gal-Transferase Knock Out</td>
</tr>
<tr>
<td>HAR</td>
<td>Hyper-Acute Rejection</td>
</tr>
<tr>
<td>HCOP</td>
<td>HGNC Comparison of Orthology Predictions</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HGNC</td>
<td>HUGO Gene Nomenclature Committee</td>
</tr>
<tr>
<td>HLHS</td>
<td>Hypoplastic Left Heart Syndrome</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human Microvascular Endothelial Cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vascular Endothelial Cell</td>
</tr>
<tr>
<td>IC</td>
<td>Inferred by Curator</td>
</tr>
<tr>
<td>IDA</td>
<td>Inferred from Direct Assay</td>
</tr>
<tr>
<td>IGI</td>
<td>Inferred from Genetic Interaction</td>
</tr>
<tr>
<td>ILT</td>
<td>InterLeaflet Triangle</td>
</tr>
<tr>
<td>IMP</td>
<td>Inferred from Mutant Phenotype</td>
</tr>
<tr>
<td>INR</td>
<td>International Normalized Ratio</td>
</tr>
<tr>
<td>ISS</td>
<td>Inferred from Sequence or Structural Similarity</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-Out</td>
</tr>
<tr>
<td>LL</td>
<td>Left-coronary Leaflet</td>
</tr>
<tr>
<td>LRP</td>
<td>low-density Lipoprotein Related Protein</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricle</td>
</tr>
<tr>
<td>LVAD</td>
<td>Left Ventricular Assist Device</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left Ventricular Ejection Fraction</td>
</tr>
<tr>
<td>LVH</td>
<td>Left Ventricular Hypertrophy</td>
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<tr>
<td>MHV</td>
<td>Mechanical Heart Valve</td>
</tr>
<tr>
<td>MLEC</td>
<td>Mouse Lung Endothelial Cell</td>
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<tr>
<td>MS</td>
<td>Membranous Septum</td>
</tr>
<tr>
<td>MV</td>
<td>Mitral Valve</td>
</tr>
<tr>
<td>NAS</td>
<td>Non-traceable Author Statement</td>
</tr>
<tr>
<td>NCL</td>
<td>Non-Coronary Leaflet</td>
</tr>
<tr>
<td>NECD</td>
<td>NOTCH ExtraCellular Domain</td>
</tr>
</tbody>
</table>
Neu5Gc | N-glycolylneuraminic acid
---|---
NGF | Nerve Growth Factor
NGS | Next-Generation Sequencing
NHP | Non-Human Primate
NICD | NOTCH IntraCellular Domain
NO | Nitric Oxide
OFT | Out-Flow Tract
PAEC | Porcine Aortic Valvar Endothelial Cells
PAVIC | Porcine Aortic Valvar Interstitial Cells
PCP | Planar Cell Polarity
PET | Positron Emission Technology
PHF | Primary Heart Field
PK15 | Pig Kidney 15 cells
PMID | PubMed ID
PTFE | Pyrolytic carbon, Teflon
Q1 | First Quartile
Q3 | Third Quartile
RL | Right-coronary Leaflet
SAVR | Surgical Aortic Valve Replacement
SD | Standard Deviation
Sda | Sd (blood group) antigen
SHF | Secondary Heart Field
SMA | Smooth Muscle Actin
ST | Sinu-Tubular
SVAS | Supra-Valvar Aortic Stenosis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVD</td>
<td>Structural Valve Degeneration</td>
</tr>
<tr>
<td>TAC</td>
<td>Trans-Aortic Constriction</td>
</tr>
<tr>
<td>TAS</td>
<td>Traceable Author Statement</td>
</tr>
<tr>
<td>TAV</td>
<td>Trileaflet or 'Tricuspid' Aortic Valve</td>
</tr>
<tr>
<td>TAVI</td>
<td>Transcatheter Aortic Valve Implantation</td>
</tr>
<tr>
<td>TAVR</td>
<td>Transcatheter Aortic Valve Replacement</td>
</tr>
<tr>
<td>TEE</td>
<td>Trans-Esophageal Echocardiography</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming Growth Factor Beta Receptor</td>
</tr>
<tr>
<td>TTE</td>
<td>Trans-Thoracic Echocardiography</td>
</tr>
<tr>
<td>UAV</td>
<td>Unileaflet or 'Unicuspid' Aortic Valve</td>
</tr>
<tr>
<td>UniProtKB</td>
<td>Universal Protein resource</td>
</tr>
<tr>
<td>VA</td>
<td>Ventriculo-Aortic</td>
</tr>
<tr>
<td>VEC</td>
<td>Vascular Endothelial Cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless/inTegrated</td>
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**Abstract:**

Aortic Valve Disease (AVD) is a progressive disease process of the aortic valve that affects an average of about 3-4% of the world population and is the most common cause of cardiac valve disease in the Western World. Recent studies have shown that AVD is, not merely a degenerative process on its own, but instead there are genetic and developmental factors that contribute to disease initiation and its progression prior to the environmental factors that add to the disease development. The current management of AVD largely relies upon aortic valve replacement (AVR), and despite treating the disease, AVR comes with a lot of risks and delayed complications. This project was aimed at: identifying the genetic pathways that are involved in aortic valve development using Gene Ontology; bridging the gap in the understanding of the development of the disease process across age groups by performing a detailed morphological analyses of stenosed aortic valves in fetal, pediatric and adult specimens; and, examining tissues from a laboratory inbred strain of pigs, the Gottingen minipig to evaluate its feasibility for use as a source of bioprosthetic valves. In order to explore the genetic pathways involved in aortic valve development, I used Gene Ontology (GO) terms to capture the role of 28 proteins to aortic valve development, 25 of which had not been previously annotated to aortic valve development in GO, by creating over 300 new annotations. In addition, 6 other proteins were annotated to other cardiac developmental processes. Secondly, in the cardiac morphology section, 56 specimens and 11 scans were studied for gross examination and high definition imaging, respectively. Standard gross morphological examination showed that there was a significant correlation between; the age of sample and site of fusion to the nature of raphe present; the nature of raphe and area of interleaflet triangle; the site of fusion and progression of severity fibrosis/endocardial
fibroelastosis (EFE); and left ventricle (LV) remodeling in relation to the fibrosis/EFE. Comparison of measurements acquired using gross examination to those obtained using a high definition novel imaging technique (micro-CT) showed that new imaging techniques allowed for the visualisation of greater detail, and more precise measurements. However, there were differences between the measurements of both techniques. Efforts to optimise technique for micro-CT imaging showed that: siliconization of the sample led to less distortion and thus, significant differences when measuring the cross-sectional area of the aorta in comparison to a non-siliconized sample; and, under- or over-iodination affected the quality of the images studied. Lastly, using a set of molecular biology techniques, tissues from two strains of pigs, were tested for the presence of xenogeneic antigens that have been shown to contribute towards the degeneration of current bioprosthetic devices. The tissues tested expressed Sda antigen and therefore, were not useful as a potential source of bioprosthetic heart valve development. Since all porcine tissues tested previously, and in this project, have xenogeneic glycans present on their surfaces, it is essential to have a standard uniform assay to measure antibody reactivity to all three xenogeneic glycans. I showed that a pig kidney cell line, PK15, expressed B4GALNT2, the gene responsible for the production of the Sda antigen. Since this cell also makes other xenogeneic antigens, Gal and Neu5Gc, this cell line could act as a uniform assay to assess antibody reactivity to all glycans. This will allow for monitoring the immune response after BHV replacement could help explore the development of long-lasting bioprosthetic devices. This thesis is the first to my knowledge that looks into aortic valve stenosis in a multifaceted approach, therefore, providing an integrative platform for future research to be carried out in the development and treatment of this disease.
**Impact Statement:**

The genetic and morphological analyses of the development of aortic valve disease and the study of the prevention of immunogenic rejection of current devices used to treat this disease, provide a unique tridimensional study that aids a greater understanding and management of the abnormalities of the aortic valve.

The graphic network of protein-protein interactions created using proteins annotated in this project has allowed for the identification of commonly shared pathways between annotated proteins and other proteins that could potentially be involved in aortic valve development, opening up avenues for further exploration into the genetic pathways in cardiac, particularly valvar, development. The results seen in the morphological section of this thesis may serve as a preamble to surgeons when faced with patients exhibiting similar phenotypes, especially preoperatively. In addition, the discrepancy seen in measurements between gross examination and imaging shows that even High Definition novel imaging techniques, such as micro-CT, may have errors, and any clinical decision taken purely on the basis of imaging should be done so with caution. Lastly, the ability of PK15 cells to express three main xenogeneic glycans that contribute towards the degeneration of bioprosthetic heart valves shows, that this assay could prove to be useful in uniformly measuring antigen-antibody reactivity of clinical bioprosthetic devices.

Together, this thesis has contributed to the field of cardiac genetics, cardiac morphology and cardiac surgery.
Chapter 1

Literature Review
Figure 1: Drawing of the heart in a coronal section.

This drawing of the heart by Frank Netter, shows the detailed anatomy of the heart in coronal plane. Note the aorta, aortic valve, mitral valve, and the left ventricle (Netter, 2016).
1.1 Overview

Cardiovascular diseases are the leading cause of death in the world claiming about 17.5 million deaths worldwide in 2012 (WHO, 2014). Valvular Heart Disease, or disease affecting the heart valves, is a major cause of cardiovascular disease affecting more than 1 million people above the age of 65 in the UK alone (Marijon et al., 2009). The human heart has two groups of valves; the atrioventricular valves, which include, mitral and tricuspid valves, and the semilunar valves, which include the aortic and pulmonary valves (Figure 1). Normal aortic and mitral valves withstand 5 to 8 folds higher transvalvular pressures than the pulmonary and tricuspid valves and are twice as thick as these right-sided heart valves (Guyton, 1976, Sacks et al., 2009). It is due to the higher pressures experienced by the left sided heart valves, that they are more prone to damage; solely accounting for over 80% of all valvular heart disease, with isolated right sided heart valve disease accounting for only around 1% of valvular heart disease. The remaining 15-20% of valvular diseases are due to multiple valve aetiologies (Iung et al., 2003, Iung and Vahanian, 2011, Nkomo et al., 2006a, Roger et al., 2011).

Aortic Valve Disease (AVD) is a progressive disease process of the aortic valve and is the most common cause of cardiac valve disease in the Western World, affecting an average of about 3 -4 % of the population, with an incidence of about 10% in those above the age of 80 years (Iung et al., 2003, Eveborn et al., 2013, Osnabrugge et al., 2013, Hufnagel et al., 2015, Bhatia et al., 2016). AVD can be used to describe both, Aortic Stenosis (AS), in which there is an obstruction to blood flow during systole due to narrowing of the aortic orifice, and Aortic Regurgitation (AR), the backflow of the blood into the left ventricle during ventricular diastole due to incomplete closure of the aortic valve (Nishimura et al., 2002, Nkomo et al., 2006b, Margulescu, 2017). The
current guidelines adopted by the American College of Cardiology/American Heart Association represent a novel concept of staging of AVD based on patient symptoms, valve anatomy and hemodynamics, and left ventricular (LV) changes, and is integral to diagnosis and management (Otto, 2015).

Table 1-1: Stages of aortic valve disease and their clinical features (adapted from Otto et al., 2015)

<table>
<thead>
<tr>
<th>Stages of Aortic Valve Disease</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage A</td>
<td>At risk for valve disease, for example, patients with aortic sclerosis or a bicuspid aortic valve</td>
</tr>
<tr>
<td>Stage B</td>
<td>Progressive valve disease, equivalent to mild-to-moderate aortic stenosis (AS)</td>
</tr>
<tr>
<td>Stage C</td>
<td>Severe asymptomatic valve disease, defined by valve anatomy and hemodynamics, with subdivisions for normal or abnormal LV function</td>
</tr>
<tr>
<td>Stage D</td>
<td>Severe symptomatic AS, including subsets for low-gradient low-flow severe AS with a low or normal left ventricle ejection fraction (LVEF)</td>
</tr>
</tbody>
</table>

AVD can be due to congenital causes, such as bi-leaflet (commonly known as bicuspid) aortic valves or acquired (Furukawa and Tanemoto, 2015, Lindman et al., 2016). Recent studies on valvulogenesis in the embryo have shown that AVD is, not merely a degenerative process on its own, but rather that there are genetic and developmental factors contributing to disease initiation and its progression prior to the
environmental factors that add to disease development (Markwald et al., 2010, Weiss et al., 2013, Yassine et al., 2017, Menon and Lincoln, 2018).

In this thesis, I aim to identify the genetic pathways involved in aortic valve development, study the morphological defects of aortic valve stenosis across the life course from fetal life to adulthood, and identify a possible novel source for development of bio-prosthetic heart valves used in both pediatric and adult age groups, that are not prone to xenogeneic structural valve degeneration (SVD) as seen in current bio-prosthetic heart valves. This study will allow a better understanding of aortic valve disease and aid in the prevention, diagnosis and treatment of aortic valve defects in the future.

In the following sections, I will review the literature on, aortic valve disease - in terms of embryology, genetics, and morphology of the normal and abnormal aortic valve and current treatment of aortic valve disease.
1.2 **Morphogenesis**

The heart is the first functional organ to develop in an embryo. There are two heart fields, the ‘primary heart field’ (PHF), formed by the migration of mesodermal cells from the anterior part of the primitive streak to the splanchnic mesoderm, and the ‘secondary heart field’ (SHF) (Figure 2) (MacGrogan et al., 2014), a group of cardiac progenitor cells located postero-medially to the former (Lawson et al., 1991, Tam et al., 1997, Abu-Issa and Kirby, 2007, Vincent and Buckingham, 2010). Cells in the PHF migrate laterally then fuse in the midline to form the heart tube, which subsequently elongates on both the arterial and venous poles via the addition of progenitor cells originating from the SHF (Figure 2) (Kelly et al., 2001, Mjaatvedt et al., 2001, Waldo et al., 2001, Cai et al., 2003).

![Figure 2: Sequence of events seen in the early stages of the development.](image-url)
(A) Ventral views of a developing mouse embryo. At day 7 (E7.0), progenitor cardiac cells reach the folds and by E7.5 have fused in the midline form two heart fields, a primary heart field (PHF) (red in color) and a secondary heart field (SHF) (blue in color). At day 8 (E8.0), the primitive heart tube elongates at the arterial and venous poles by addition of SHF cells. The heart tube is then suspended from the splanchnic mesoderm by the mesocardium (not shown). Between E8.0 and E9.0 the heart tube loops. (B) Ventral view of the mouse heart at E9.5. the heart has four anatomically separate regions: the Atrium (At), the Outflow Tract (OFT), the Atrioventricular Canal (AVC), and the ventricle (V). (C) Longitudinal view of the mouse heart at E9.5. Presence of elongated spiralling cushions in the OFT can be seen – divided into proximal, intermediate, and distal parts. There are 4 cushions present in the AVC, the right lateral (rlAVC), the left lateral (llAVC), superior (AVC), and the inferior (AVC) (reproduced from MacGrogan et al., 2014).

The developing heart tube is formed by endocardial cells that come from both sides of the developing embryo and are then surrounded by an outer layer of myocardial cells. This outer layer of cells does not cover the lumen of the primary heart tube in its entirety, and is, in fact, suspended - in its dorsal aspect - from the body wall (splanchnic mesoderm) by a structure called the dorsal mesocardium. The heart tube further develops by addition of migratory cells at its cranial and caudal poles. The dorsal mesocardium, which initially connected the developing left ventricle to the mediastinum, is disrupted allowing for the large tube to be unattached. The dissolution of the dorsal myocardium except at the poles of heart tube frees the tube to bend (rightward); a phenomenon called ‘looping’ (Vincent and Buckingham, 2010, MacGrogan et al., 2014).

As a result of looping, the primary heart tube within the pericardial cavity can be divided into atrial and ventricular components along with an outflow tract (Figure 2B). The developing atria and ventricles are separated by the atrioventricular canal, and the developing outflow tract is supported by the distal part of the ventricular loop. After looping, the tube has an inner and outer curvature (MacGrogan et al., 2014, Anderson et al., 2016). The apices of both ventricles balloon form the outer curve, with the inner curve realigning so that each apex of the ventricle has an inlet and an outlet part
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(Anderson et al., 2016). Studies conducted on ancestor chordate hearts and the embryonic hearts of higher vertebrates have demonstrated that the hearts consist of ‘pacemaker like’ cardiac muscle cells with maximal pacemaker activity at the ventral end signifying presence of a one direction peristaltic contraction activity (Simoes-Costa et al., 2005, Anderson et al., 2016). Presence and interconnectivity of such well-developed embryonic myocardial cells with low pacemaker activity permit quick and efficient impulse conduction and contraction which leads to the gradual proliferation of the myocardium and formation and positioning of the heart’s conduction system, that results in a sequential and regionalized chamber formation (Moorman et al., 2003).

1.2.1 Formation of Endocardial Cushions

As the tube loops, important changes take place within the hyaluronin-rich extracellular matrix (between the endocardium and myocardium), called cardiac jelly (Rozario and DeSimone, 2010, Lockhart et al., 2011, de Vlaming et al., 2012). Accumulation of the cardiac jelly in the lumens of the AVC and proximal OFT give rise to local tissue swellings, termed endocardial cushions (Figure 2C) (Lin et al., 2012, Bolar et al., 2017). Stimulated by signals sent by the developing myocardium, endocardial (specialized epithelial) cells overlying the endocardial cushions undergo a process called endothelial-to-mesenchymal transition (EMT) which results in transformation of endothelial cells into mobile mesenchymal cells (de Vlaming et al., 2012, Kovacic et al., 2012, von Gise and Pu, 2012). A similar process is seen within the lumen of the outflow tract (OFT), where alongside EMT, the addition of cells derived from the neural crest converts the, initially acellular, cardiac jelly within the outflow tract into tissue swellings, termed truncal cushions (MacGrogan et al., 2014, Anderson et al., 2016).
There are three key stages to EMT: activation, invasion and migration, which eventually results in relatively bulky and cellularised endocardial cushions (Eisenberg and Markwald, 1995, Barnett and Desgrosellier, 2003, Lincoln and Garg, 2014) (Table 1-2).

### Table 1-2: Stages of EMT (adapted Barnett and Dresgrosellier 2003)

<table>
<thead>
<tr>
<th>Stages of EMT</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>Endothelial cells of the endocardium separate and adopt a mesenchymal phenotype</td>
</tr>
<tr>
<td>Invasion</td>
<td>Penetration of mesenchymal cells into the cardiac jelly</td>
</tr>
<tr>
<td>Migration</td>
<td>Migration of mesenchymal cells deeper into the cardiac jelly</td>
</tr>
</tbody>
</table>

Fusion and maturation of the endocardial and truncal cushions along with mesenchymal tissue on the atrial septum and the dorsal mesenchyme is responsible for the division of the AVC and the OFT. The separation of the AVC into left (mitral) and right (tricuspid) AV junctions forms ventricular inlets that connect the respective atrium to the ventricle, whereas, the outflow tract divides into the left and right ventricular outlets that connect the left and right ventricle, respectively, to the developing aorta and pulmonary trunk. The OFT endocardial cushions give rise in their intermediate part to semilunar (aortic and pulmonary) valves (Fraisse et al., 2003, de Lange et al., 2004).

### 1.2.2 Aortic Valve Development

Endocardial cushions with mesenchymal cells elongate and remodel themselves to form primitive valves that gradually mature into thin valve leaflets. The elongation of
valve leaflets is accomplished by a combination of cell proliferation at the growing edge and apoptosis at the base of the cushion (Figure 3) (Hurle et al., 1980, MacGrogan et al., 2014).

Figure 3: Epithelial-to-mesenchymal transformation (EMT) and valve elongation.

Endocardial cells in the AV cushions and outflow tract cushions undergo EMT and give origin to mesenchymal cells that migrate into the cardiac jelly and populate the cushions. The mesenchymal cushions then remodel and elongate themselves to form primitive valves that mature into thin valve leaflets (shown here for the atrioventricular valves and the semilunar valves respectively) (reproduced from Lin et al., 2012).

The semilunar valves and their supporting sinuses develop from the ‘distal’ and ‘intercalated’ cushions of the OFT. The distal cushions give rise to the right and left leaflets of each of the valves; in the aorta, these form the right and left coronary leaflets, whereas, in the pulmonary valve, they are the right and left leaflets (de Lange et al., 2004, Restivo et al., 2006, Okamoto et al., 2010). The right-posterior and the left-anterior intercalated cushions form the non-coronary leaflet in the aorta, and the anterior pulmonary leaflet, respectively (Anderson et al., 2003, Restivo et al., 2006). The valvar sinuses are formed through apoptosis and alterations in the extracellular matrix by contributions of non-myocardial tissues from the SHF (Martin et al., 2015,
Anderson et al., 2016). This results in a formation of the central lumen of each cushion that separates the three valve leaflets, with the peripheral portion arterializing to form the wall of the supporting valve sinuses. Valvulogenesis continues with elongation and thinning of the endocardial cushions by remodeling and compartmentalizing into the collagen-rich fibrosa, proteoglycan-rich spongiosa, and elastin-rich atrialis/ventricularis layers (Figure 3) (Gomez Stallons et al., 2016, Ayoub et al., 2016, Hinton et al., 2006). The extracellular matrix composition and organization of the valve leaflets are critical for normal valve function, and dysregulation of extracellular matrix remodeling or structural components can lead to valve malformations (Brickner et al., 2000, Combs and Yutzey, 2009).

Understanding normal valve development is fundamental to gain an insight into the structural development of the malformed aortic valve and the manifestation of the aortic valve disease.
1.3 Genetic Pathways involved in Aortic Valve Development

Many studies have shown that a large proportion of patients who acquire aortic valve disease (AVD) have congenital valve malformation, indicating that in many cases there is a genetic and developmental basis of adult AVD (Roberts and Ko, 2005, Pierpont et al., 2007, Wirrig and Yutzey, 2014). In order to understand the development of AVD, research has focused on the identification of the genetic pathways and systems that are involved in the development of aortic valves. Mouse and human studies have identified causative gene mutations, using specific mouse models, next generational sequencing and genetic linkage studies, for congenital aortic valve malformations, such as bicuspid/bileaflet aortic valve (BAV), and adult AVD, such as calcific AVD (CAVD) (Bonachea et al., 2014). This has led to the identification of over 40 genes associated with the genetic causes of AVD many of which have previously known roles in valve development (Wu et al., 2017). These genes encode proteins important in two key processes: (1) signaling pathways and regulation of transcription factors, which initiate cellular processes involved in valve development, and (2) structural composition and organization of valve extracellular matrix (ECM).

One of the main cellular processes that takes place in valvulogenesis is endothelial to mesenchymal transformation (EMT) that gives rise to a population of highly proliferative and migratory valve precursor cells. Many signaling pathways have been found to be associated with EMT. A relatively small set of highly evolutionarily conserved signaling pathways, however, stand out as having critical roles during valve development: NOTCH signaling pathway, TGF-beta signaling pathway and WNT signaling pathway. These signaling pathways are inter-connected with one another to form a complex genetic network between differential cell types (Figure 4).
Figure 4: The co-regulation of various signaling pathways that are involved in heart valve development and remodeling.

The signaling network demonstrates the numerous pathways and transcriptional regulators that act in a coordinated manner to regulate the process of heart valve formation. Each signaling pathway shown is indicated by the key protein the pathway is named after, thus providing a simplified schema of the signaling events that occur. Red arrows denote positive/synergistic interactions between pathways. Blunt red arrows denote inhibitory effects between pathways (reproduced from Ehrin J. Armstrong, and Joyce Bischoff Circulation Res. 2004; 95:459-470).

Many growth factors produced by developing cardiomyocytes and endothelial cells contribute to the EMT. Some of the main growth factor receptors responsible for the initiation of this process are NOTCH family members, found predominantly in the valve endothelial cells (Timmerman et al., 2004, Macgrogan et al., 2011, Luxán et al., 2016) and Transforming Growth Factor-beta (TGFβ1-3), found in the myocardium (Nakajima et al., 2000, Person et al., 2005b). Both, NOTCH and TGF-beta signaling pathways regulate many transcription factors, such as HEY, SNAIL and SOX9 which play a role in regulating the proliferation of newly transformed cells and remodeling.
of the matrix (Akiyama et al., 2004, Lincoln et al., 2007). Other signaling pathways, including the WNT signaling pathway (Cai et al., 2013) play a role in angiogenesis as part of the remodeling and shaping of the endocardial cushions into valve leaflets during later stages of development (Van Nieuwenhoven et al., 2017). As fetal life progresses (and after birth), cardiac valves stratify into highly organized collagen, elastin and proteoglycan extracellular matrix (Rabkin-Aikawa et al., 2004). This structural framework of the valve, including a number of cell-cell and cell-matrix interactions, is formed under the influence of structural proteins, e.g. Cartilage Intermediate Layer Protein, and several genetic signaling pathways (Gittenberger-De Groot et al., 2005).

In order to further explore the roles of these genetic pathways, studies have mainly been carried out in animal models e.g. mouse and chick, due to the ethical restraints of studying development in humans. Due to the evolutionary relation of the mammalian heart, and the close shared genetic orthology between murine genes and human genes, the review that follows is restricted to mouse models only.

1.3.1 Notch Signaling Pathway

NOTCH proteins are a family of transmembrane proteins that form the core component of the namesake, NOTCH signaling pathway. This pathway is a highly conserved, fundamental signaling pathway that regulates cell fate specification, differentiation, and tissue patterning through intercellular interactions (Perrimon et al., 2012). NOTCH proteins act as single-pass trans membrane receptors and consist of, a large (NOTCH) ExtraCellular Domain, NECD, and a (NOTCH) IntraCellular Domain, (NICD), with two nuclear localization signals and a transactivation region (Kovall et al., 2017) (Figure 5).
NOTCH is processed in the Golgi apparatus by proteolytic cleavage, catalyzed by a furin-like convertase, and, addition of sugars to the NECD by glycosyl transferases (Luxán et al., 2016). The modified NOTCH is then directed to the cell surface as a heterodimer bound together by non-covalent interactions (Macgrogan et al., 2011). Once NOTCH is in the membrane, the NECD region interacts with any one membrane-bound ligands, of the Delta (DLL1-DLL4) and Jagged (JAG1, JAG2) proteins, located in the membrane of adjacent cells.

The ligand-receptor interaction leads to conformational changes in NOTCH exposing its proteolytic cleavage site. An ADAM-family metalloprotease called ADAM10, cleaves the NOTCH protein just outside the membrane, causing separation of the extracellular domain of NOTCH (NECD) from the NICD. The NECD, still bound to the ligand, is endocytosed by the ligand-expressing cell. The remaining part of the

Figure 5: The NOTCH signaling pathway ligands and the tri-domain receptor (NOTCH).

The Notch Ligands (DLL1, 3, 4 and 5 or Jag1-2) are transmembrane proteins located in the plasma membrane of an adjacent cell. The Notch signaling pathways is initiated in the Notch Receptor containing cells.
receptor undergoes a second proteolytic cleavage by gamma secretase activity, resulting in the release of the NICD from the transmembrane domain, and the NICD then translocates to the nucleus of the signal receiving cell (Figure 6) (Logeat et al., 1998, Le Borgne et al., 2005).

**Figure 6: NOTCH signaling pathway.**

The ligand binds to the tri-domain NOTCH receptor, and causes S2 cleavage of the NOTCH receptor, with the separation of the Notch ExtraCellular Domain (NECD still bound to ligand) from the other two domains. This is followed by another cleavage where the NOTCH IntraCellular domain (NICD) separates from the Transmembrane Domain, and translocates to the nucleus where it binds to RBPJ and initiates transcription of target genes such as HEY and SNAIL.

In the nucleus, the NICD binds to the transcription factor, recombination signal binding protein for immunoglobulin kappa J region (RBPJ neé RBPJK), forming a complex that activates the expression of target genes, which include those encoding
basic-helix-loop-helix transcription factors of the HES and HEY families (Figure 6) (Le Borgne et al., 2005, Tabaja et al., 2017).

**Clinical significance of NOTCH signaling pathways**

A relatively simplistic signaling pathway, in terms of molecular design, the NOTCH signaling pathway is involved in a wide range of processes during embryonic and adult life, by regulating organogenesis and critical cellular processes such as proliferation and apoptosis (Lai, 2004). NOTCH has been identified to play a role in several developmental processes, such as neuronal function and development, pancreatic development, and cardiovascular development (Gaiano and Fishell, 2002, Murtaugh et al., 2003, Grego-Bessa et al., 2007, Aguirre et al., 2010). Variants of several NOTCH family members and some of their ligands have been shown to result in a number of congenital human diseases, including some forms of AVD (Garg et al., 2005). The role of the NOTCH signaling pathway in valve development has been further explored using *ex vivo* and *in vitro* mouse models.

**1.3.1.1 Role of NOTCH signaling pathways in valve development**

Studies looking at expression patterns of NOTCH signaling proteins have found them to be present in the heart implying the crucial role of the NOTCH signaling pathway in early heart development. Due to the involvement of the pathway in multiple aspects of development, complete knockout models show early embryonic lethality, which makes it difficult to study the role of Notch in valve development, specifically; making it essential to generate tissue specific knockout mice.

The expression of specific ligands, receptors and target genes, at certain sites within the heart suggests a role of Notch in the developmental processes occurring within that
tissue during a particular time period. Table 1-3 shows the various sites of expression for different components of the Notch signaling pathway.

**Table 1-3: Components of Notch signaling pathway and their expression sites in mouse embryos.**

<table>
<thead>
<tr>
<th>Components of Notch signaling pathway</th>
<th>Expression sites in mouse embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ligands</strong></td>
<td></td>
</tr>
<tr>
<td>Dll4</td>
<td>endocardium of the primitive heart tube and the ventricular endocardium</td>
</tr>
<tr>
<td>Jag1 and Jag2</td>
<td>Atrioventricular canal (AVC) and outflow tract (OFT) endocardium and atrial myocardium</td>
</tr>
<tr>
<td><strong>Receptors</strong></td>
<td></td>
</tr>
<tr>
<td>Notch1</td>
<td>cardiac mesoderm, endocardium – especially in AVC and OFT, and cardiac cushion mesenchyme</td>
</tr>
<tr>
<td>Notch2</td>
<td>AVC and OFT endocardium and cushion mesenchyme and is later on expressed in atrial and ventricular myocardium</td>
</tr>
<tr>
<td>Notch3</td>
<td>cardiac mesoderm</td>
</tr>
<tr>
<td>Notch4</td>
<td>cushion formation</td>
</tr>
<tr>
<td><strong>Target Genes</strong></td>
<td></td>
</tr>
<tr>
<td>Hey1</td>
<td>primitive heart tube, and atrial myocardium</td>
</tr>
<tr>
<td>Hey2</td>
<td>primitive heart tube, ventricular myocardium, and AVC and OFT endocardium.</td>
</tr>
</tbody>
</table>
The NOTCH family and their ligands

In mammals, there are four members of the NOTCH family, NOTCH1-4, and five different ligands, Dll1, Dll3, Dll5, Jag1 and Jag2 which can bind to these receptors to initiate the NOTCH signaling pathway, (Kumar et al., 2016). In order to examine the effects of the loss of specific components of Notch signaling during murine development, ligand and receptor specific transgenic mouse have been generated.

Knockout of Notch ligands, such as, Jag1 in mouse embryos have resulted in abnormal migration of cardiac neural crest cells, defective EMT, and vascular defects, such as collapsed vasculature and haemorrhaging amongst others (Hofmann et al., 2012), leading to embryonic lethality at E10.5 (Garside et al., 2013).

Similarly, various cardiac defects are seen when Notch is knocked out in mice. Notch1 -/- mice have collapsed endocardium and lack cushion mesenchyme at the onset of valvulogenesis and also result in embryonic lethality at E10.5, signifying the vital role of Notch during early phases of development (Timmerman et al., 2004). Furthermore, tissue specific deletion of Notch2 in neural crest cells, albeit not exhibiting the valvar defects seen with the deficiency of Notch1, result in a constricted outflow tract due to decreased proliferation of the vascular smooth muscle cells, causing ventricular hypoplasia and decreased trabeculation within the myocardium of the ventricle. The presence of healthy valves in Notch2 -/- mice is indicative of compensatory mechanisms by other Notch family members such as Notch1, providing evidence that Notch1 is essential for cardiac valve formation and suggesting the role of Notch an important mediator of interactions between second heart field, cardiac neural crest cells, and outflow tract (OFT) endothelium (De la Pompa and Epstein, 2012).
NOTCH co-factors and target genes

Transgenic mice have also been generated for the downstream intracellular effectors of the Notch signaling pathway, such as NICD and Rbpj. Examination of the hearts from these mice has shown that the loss or overexpression of these intracellular effectors can lead to a complete block or constitutive activation of Notch signaling, respectively. For example, a deficiency of Rbpj causes a loss of cushion mesenchyme in valve regions, EMT defects, and collapsed endocardium in the developing heart (Timmerman et al., 2004, Garside et al., 2013). In Rbpj-/- mouse embryos, the endocardium overlying AVC cushions fail to invade the cushions due to preservation of these cells’ adherens junctions which are responsible for the maintenance of close intercellular associations and prevent movement of cells. In the presence of Rbpj, these junctional complexes are locally disassembled in order for EMT to occur in the outflow tract (Timmerman et al., 2004). As Rbpj is a downstream mediator of Notch signaling, this result suggests that Notch activation and signaling via Rbpj is essential for the EMT in the cushions.

A variety of mouse studies have further strengthened the notion that the Notch signaling pathway, via the Hey family of transcription factors, plays a significant role during cardiac valve development. For example, a double knockout of the Notch target genes, Hey1 and HeyL shows failure of closure of ventricular septum and thickening of valve leaflets (Fischer et al., 2007). Whereas, Hey2 -/- mice exhibit a similar phenotype to patients with tetralogy of Fallot (Donovan et al., 2002). In addition, Kokubo et al. showed that the Notch target genes Hey1 and Hey2 in the atrial and ventricular chambers are involved in restricting Bmp2 and Tbx2 expression in the AVC and OFT (Kokubo et al., 2007). As a deficiency of murine Bmp2 and Tbx2 leads to
AVC defects (Harrelson et al., 2004, Ma et al., 2005), the restriction of *Bmp2* and *Tbx2* may be one of the mechanisms by which these Hey proteins affect heart development.

The aforementioned studies imply that NOTCH receptors play an essential role in the epithelial to mesenchymal transition, a key stage in heart valve development.

1.3.1.2 The role of the NOTCH signaling pathway in valve disease development

Valve calcification is a common component of heart valve disease. *In vitro* models using aortic valve interstitial cells (AVICs) have provided insights into the basis of calcific valve disease. The AVICs display osteoblast-like characteristics and are present, along with endothelial cells and a structurally rigid extracellular matrix (ECM), within a mature adult valve. A disruption in the organization of the ECM results in abnormal valvar development and disease. The involvement of Notch signaling in the regulation of multiple molecular pathways during embryonic life has led to the exploration of the role of Notch in adult life. Notch1 and its downstream mediators are expressed in an adult aortic valve and loss of Notch signaling is associated with areas of calcification in human aortic valves (Luxán et al., 2016). Furthermore, inhibition of Notch signaling has resulted in significant changes in the expression of cartilage specific genes that are responsible for maintaining the ECM. Studies on zebrafish models have provided evidence that endocardial Notch signaling mediates fibrotic tissue and cardiomyocyte proliferation within the valve (Raya et al., 2003, Gonzalez-Rosa et al., 2011, Zhao et al., 2014). These findings suggest that endocardial Notch1 is required for the activation of anti-osteogenic molecules and inhibition of the inflammatory genes within the adult valve. Gene analyses on human patients suffering from bicuspid/bileaflet aortic valve (BAV) have provided evidence that mutations in *NOTCH1* can be linked with familial, non-syndromic autosomal
dominant calcific aortic valve disease (CAVD) and BAV (Mohamed et al., 2006, McBride et al., 2008). Although, a greater understanding of the role of Notch in calcification is yet to be explored, the studies above provide sufficient evidence that the Notch Signaling pathway does play a role in adult aortic valve disease and valvar calcification.

1.3.2 TGF-beta Signaling Pathway

TGF-beta (Transforming Growth Factor beta) is the prototypic member of a growth factor superfamily, with 33 members in human including: three TGF-betas (TGFB1-3), five inhibins (with the root symbol INH), 11 bone morphogenetic proteins (BMP1-7, 8A, 8B, 10, 15) amongst others (Heldin and Moustakas, 2016). These growth factors are involved in the majority of developmental processes, not just heart development. The generic (biological) functions of TGF-beta can be broadly classified into three major categories: (1) affecting cellular proliferation and migration, (2) involvement in cell adhesion and ECM formation, and (3) affecting a cell’s phenotype (Massague, 1990, Roberts and Sporn, 1993). The cellular impact of the TGF-beta signaling pathway depends on the following: the extracellular concentration of TGF-beta, the binding of TGF-beta as a ligand to a cell-surface receptor, interaction of TGF-beta ligands with accessory receptors, regulatory signals from other signaling pathways and the set of target genes regulated. Disruptions in the TGF-beta signaling pathways are often associated with developmental disorders and diseases (Zhu and Burgess, 2001, Massague, 2012).

The TGF-beta signaling pathway begins with the secretion of TGF-beta. These ligands are disulphide-linked dimers, that act as paracrine factors on cells local to the source (Wrana, 2013). TGF-beta then binds to one of many TGF-beta receptors (TGFBRs).
TGFBRs are dual specificity transmembrane receptor kinases which form heterotetrameric complexes of two type I and two type II receptors on ligand binding. There are three types of TGFBRs receptors categorized as type I (signal propagator), type II (activator), or type I-II (regulatory/accessory receptor) (Heldin and Moustakas, 2016). There are two pathways through which TGF-beta signaling can take place, (1) canonical, and (2) non-canonical (Wrana, 2013).

**Canonical Pathway**

The canonical pathway of TGF-beta signaling is based on the involvement of SMADs, a family of structurally similar intracellular signaling proteins. TGF-beta binding to transforming growth factor beta receptor 2 (TGFBR2), leads to the activation of transforming growth factor beta receptor 1 (TGFBR1) through formation of a heteromeric complex (Zhu and Burgess, 2001) (Figure 7). This results in the phosphorylation of both the receptor-regulated SMAD proteins (R-SMADs; SMAD2 and 3) in the intracellular juxta-membrane region (Moustakas et al., 2001). Once the R-SMADs are phosphorylated, they interact with the common partner (coSMAD), SMAD 4, to form the R-SMAD/coSMAD complex. This complex then translocates to the nucleus where the complex then interacts with DNA binding cofactors, coactivators, and co-repressors to regulate mRNA transcription (Garside et al., 2013). The TGF-beta signaling pathway is also regulated by presence of inhibitory SMAD proteins (I-SMADs; SMAD6 and 7), which inhibit the activation of R-SMADs, as part of a negative feedback loop (Figure 7) (Zhang, 2009).

**Non-Canonical Pathway**

There are several non-canonical TGF-beta signaling pathways (Moustakas and Heldin, 2009, Zhang, 2009), one of which is necessary for EMT and based on the activation
of the MAPK pathway (Gui et al., 2012). Binding of the TGF-beta to the TGFBRs induces dimerization and activation of the TGFBR. This results in phosphorylation of the tyrosine residues found within these receptors, which then serve as binding sites for several signaling molecules, including MAP3K7 née TAK1 (a mitogen-activated protein kinase) and growth factor receptor binding protein 2 (GRB2) (Figure 7). GRB2 interacts with ‘SOS’, and TGFBR activation results in the transport of the GRB2/SOS complex to the plasma membrane. The SOS molecule then activates a RAS type GTPase family member, which in turn binds to a RAF kinase, resulting in activation of a MAPK cascade. The activated Erk is then translocated to the nucleus. Erk activation is one of the non-Smad pathways necessary for TGF-beta-mediated EMT (Davies et al., 2005, Zavadil et al., 2006, Zhang, 2009) (Figure 7).

![Figure 7: TGF-beta signaling pathway.](image-url)
The binding of TGF-beta ligands to TGF-beta receptors initiates the signaling pathway. In the canonical pathway, the ligand-receptor interaction causes phosphorylation of SMAD2 and 3. The phosphorylated SMAD2 and 3 then bind to SMAD4, and the complex translocates to the nucleus and causes transcription of target genes i.e. GATA4 and GATA5. In the non-canonical pathway on the other hand, the same ligand-receptor interactions are involved but, in this case, the dimerized and activated receptor serves as binding sites for signaling molecules such as TAK1, TAB1, and GRB2. GRB2 interacts with the SOS complex which after a series of other interactions activates ERK. ERK is then responsible for inhibiting GSK3B which in turn inhibits SMAD3 and 4, thereby reducing canonical TGF-beta signalling (adapted from Davies et al., 2005, Zavadil et al., 2006 and Zhang et al., 2009).

Clinical significance of TGF-beta signaling

The functional diversity of members of the TGF-beta family can be noted by overlapping and distinct spatial and temporal patterns of expression throughout development and in adult life, with pronounced embryonic expression in areas undergoing morphogenesis (Pelton et al., 1991, Azhar et al., 2003). This has led to the identification of the role of individual TGF-beta superfamily members in the regulation of many different developmental processes, such as cardiac development, vascular development, and connective tissue development, amongst others (Gordon and Blobe, 2008).

Knockout mice for members of the TGF-beta superfamily display non-overlapping phenotypes in most major organ systems indicating a high degree of functional specificity (Sanford et al., 1997, Dünker and Krieglstein, 2000). One of these organ systems is the heart. Genetic mutations in TGF-beta pathway genes and TGF-beta signaling dysregulation have emerged as a major molecular pathway involved in adult cardiovascular diseases (Ten Dijke and Arthur, 2007, Jain et al., 2011). A large number of these cardiovascular defects such as, aortic aneurysm, aortic stenosis (AS), pulmonary hypertension and hypertrophic cardiomyopathy, are modeled in mice with mutations or dysregulation in TGF-beta signaling and effector genes (Arthur and Bamforth, 2011b, Gordon and Blobe, 2008, Heldin and Moustakas, 2016).
1.3.2.1  Role of TGF-beta signaling pathways in valve development

The TGF-beta signaling pathway is known to play an important role in the development of the endocardial cushions and their derivative structures (cardiac septa and cardiac valves) (Engelmann et al., 1992, Azhar et al., 2003). In humans, an increased expression of TGF-beta and other members of the TGF-beta superfamily, in the ECM, has been found in calcified valve cusps compared to noncalcified cusps both in vitro and ex vivo (Jian et al., 2002, Kaden et al., 2004, Clark-greuel et al., 2007, Yanagawa et al., 2012). Furthermore, TGFB1 has been associated with myocardial remodeling in patients with AS and mice models (Bjørnstad et al., 2008, Villar et al., 2009, Xu et al., 2010). These findings suggest the role of the TGF-beta signaling pathways in valve development and disease. Due to the multigenic nature of these diseases, identification of one gene in one signaling pathway, might lead to identification of other genes involved in valve disease, due to an association with the same pathway.

TGF-beta ligands and receptors

There are three mammalian TGF-beta ligand isoforms, TGFB1, TGFB2, and TGFB3 which are encoded by three separate genes, TGFB1, TGFB2, and TGFB3 (Heldin and Moustakas, 2016). All three are expressed in early heart development and our knowledge of their role in valve development is primarily based on mouse knockout studies (Azhar et al., 2009). Although all three of these growth factors are expressed in early mammalian heart development, each appears to have a specific and essential role in the development of this structure. Although the levels of each specific ligand may be more detectable within a limited time frame in a specific area, TGF-beta ligands show dynamic and frequently overlapping expression.
Tgfβ1 is expressed throughout the mouse endocardium from E8.0, however, expression becomes restricted to the atrioventricular (AV) endocardium, and then limited in vascular endothelial cells (VECs) until just after birth (De la Pompa and Epstein, 2012). Homozygous Tgfβ1−/− mice showed valvar abnormalities suggesting Tgfβ1 is involved in valvular development from the endocardial cushions (Engelmann et al., 1992).

Tgfβ2 is expressed in the mouse AVC endocardium and myocardium, and cardiac cushion mesenchymal cells from E10.0. Tgfβ2−/− mice exhibited numerous defects in the OFT cushions, AVC and cardiac septa (Jiao et al., 2006), which provides evidence for the involvement of Tgfβ2 in the development of endocardial cushions derived structures. Further in vitro studies in Tgfβ2−/− mice have shown hypoplastic endocardial cushions, in early development, and hyperplastic cushions and valves during later stages of development, suggesting that Tgfβ2 also plays a regulatory role, in these regions of the heart, and is involved in both initiation and cessation of EMT (Molin et al., 2003, Azhar et al., 2009).

Tgfβ3 is expressed in endocardial cushion mesenchyme at E11.0 after EMT has taken place, with no expression seen in the endocardium. Studies on Tgfβ3−/− mice, however found no valvular defects, suggesting that, unlike other members of the TGF-beta family, Tgfβ3 does not play a role in endocardial cushion development (Boyer et al., 1999, Molin et al., 2003).

As discussed previously there are three TGF-beta binding receptors; the TGF-beta binding receptor complex being a homodimer, heterodimer or heteromeric complex of these receptors (Heldin and Moustakas, 2016). TGFBR1 is expressed in the mammalian myocardium, endocardium and cushion mesenchymal cells (De la Pompa
and Epstein, 2012). Studies showed that targeted knockout of the Tgfbr1 in the mouse endocardium resulted in severely hypoplastic AV cushions, and in vitro Tgfbr1 -/- endothelial cells failed to undergo EMT, providing evidence that Tgfbr1 plays an important role in EMT (Arthur and Bamforth, 2011a, von Gise and Pu, 2012). Similar effects were not seen in endocardial cushions present in the OFT, suggesting that there could be different regulatory mechanisms in these cells.

TGFBR2, like TGFBR1, is also expressed in the mammalian myocardium, endocardium, and cushion mesenchymal cells. Studies show that the effect of the knockout of Tgfbr2 on EMT is dependent on whether the gene expression was downregulated in vitro or in vivo (Lebrin et al., 2005, von Gise and Pu, 2012). Endocardium-specific knockdown of Tgfbr2 did not affect EMT in vivo although it did lead to a decrease in mesenchymal cell proliferation in the inferior cardiac cushion, whereas the same knockdown was shown to block EMT in vitro. Further studies demonstrated that knockdown of Tgfbr2 leads to failure of cushion fusion which could lead to valvular defects (Robson et al., 2010, Kloesel et al., 2016).

The TGF-beta 3 receptor (TGFBR3) is an accessory transmembrane receptor with affinity for each of the three TGF-beta ligand isoforms. TGFBR3 is more than a TGFB co-receptor and can act as a down-regulator of TGF-beta signaling, by binding with scaffolding proteins and initiating signaling through G protein–independent pathways, such as MAPK signaling, instead of binding to TGFB ligands and initiating the TGF-beta signaling (Heldin and Moustakas, 2016). TGFBR3 is expressed at low levels in AVC endocardium (De la Pompa and Epstein, 2012). Although, the role of TGFBR3 in endocardial cushion development has not been fully elucidated, studies show that Tgfbr3 -/- mice are embryonic lethal by E18.5 and exhibit a wide array of cardiac
defects, albeit normal endocardial cushion development (Arthur and Bamforth, 2011a, Briggs et al., 2012).

Due to the structural similarity between the human and mouse genes, based on the experimental data above on Tgfb1-3 and Tgfbr1-3, one can infer similar roles of human TGFβ1-3 and TGFBR1-3.

**SMADs**

SMADs are the downstream signal transducers in the TGF-beta canonical signaling pathway. There are 5 receptor SMADs (R-SMADs); SMAD 1, 2, 3, 5 and 9 (alias SMAD8), each of which bind to the single common mediator SMAD, or coSMAD, SMAD4, to form the R-SMAD/coSMAD complex. Activation of the R-SMADs varies depending on the type of receptors involved; BMP type I receptors canonically activate the SMAD1/5/8, whereas, TGF-beta type I receptors activate SMAD2/3 (Kretzschmar and Massague, 1998, Wharton and Derynck, 2009).

Although, BMP signaling is involved in heart development, as evident by valvar defects seen in BMP-dependent Smad1, 5 or 8 knockout embryos (Arnold et al., 2006) and the role of BMPs in the activation of transcription factors augmenting cardiac differentiation, such as GATA4, NKX2.5, and MEF2C (Duan et al., 2017), these pathways have not been the focus of this project.

TGF-beta dependent SMAD 2 and 3, on the other hand, have a more distinct role in the development of valvar disease (discussed below in section 1.3.2.2). For example, Smad 2 −/− mice result in embryonic lethality, due to failure to establish an anterior-posterior axis, gastrulation and mesoderm formation, and they also exhibit abnormal
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It is important to appreciate the convergence of both the BMP and TGF-beta pathways at the co-SMAD, SMAD4, when considering the role of this SMAD in cardiac development (Azhar et al., 2010). Consequently, endocardial specific deletion of murine Smad4 results in embryonic lethality by E10.5 due to a wide array of cardiovascular defects, including enlarged heart, absent or acellular endocardial cushions in the AVC and OFT and failure of EMT, suggesting that SMAD4 plays a key role in all of these processes (Lan et al., 2007, Moskowitz et al., 2011).

Further downstream, inhibitory SMADs (I-SMADs), SMAD6 and 7, operate a negative feedback loop regulating the TGF-beta signaling pathway, by suppressing R-SMADs. Where SMAD7 is involved in inhibiting both BMP and TGF-beta signaling pathways, SMAD6 is restricted to the inhibition of the BMP signaling pathways (Yan et al., 2016, Miyazawa and Miyazono, 2017).

1.3.2.2 Role of TGF-beta signaling pathways in cardiac disease

TGF-beta dependent SMAD2/3 upregulation has been seen in different pathophysiological conditions and is closely correlated to increased collagen type I expression (Hao et al., 1999, Yang et al., 2015). Myocardial changes resulting from pressure overload in conditions such as transaortic constriction (TAC), activates Smad2/3 and ERK1/2 within endothelial cells in cardiac blood vessels (Wei et al., 2013). Smad3-/- mice, exhibit a 60% decrease in myocardial fibrosis and a significant increase in cardiac hypertrophy, implying that Smad3 mediated signaling pathways may play dual roles in the heart: delimiting hypertrophic growth and modulating myocardial fibrosis (Duan et al., 2017, Khalil et al., 2017).
1.3.3 WNT Signaling Pathway

The WNT (Wingless/iNTegrated) signaling pathways are an evolutionary conserved group of pathways responsible for a wide array of cellular processes, including cell fate, determination, motility, polarity and organogenesis (Komiya and Habas, 2008). There are 14 human WNT (WNT1-11 and WNT14-16) genes encoding the secreted WNT glycoproteins that bind to the N-terminal extracellular cysteine-rich domain of the frizzled (FZD) family receptors and low-density lipoprotein related protein (LRP) co-receptors (Figure 8). Upon binding to the receptor complex, WNT transduces a signal to a member of the cytoplasmic phosphoprotein disheveled (DVL) family stimulating multiple intra-cellular signal transduction cascades including the canonical and the non-canonical pathways (Katoh and Katoh, 2007, Komiya and Habas, 2008).

**Canonical Pathway**

The canonical pathway is the WNT pathway that causes an accumulation of beta-catenin (CTNBB1) in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional co-activator of transcription factors belonging to the TCF/LEF family (Yokoyama et al., 2007).

In the absence of WNT, CTNBB1 is phosphorylated within this complex by casein kinase 1 alpha 1 (CSNK1A1), and is thereafter, targeted by GSK3 for ubiquitination and subsequently proteolytic destruction (Katoh and Katoh, 2007) (Figure 8).

In the presence of WNT, WNT binds to FZD, and the transmembrane co-receptors LRP5 and 6 (low density lipoprotein related protein 5 and 6), inducing the translocation of an important signaling regulator, AXIN, to the membrane. Upon membrane translocation, AXIN binds the GSK3 (glycogen synthase kinase 3) phosphorylated LRP5/6 resulting in the activation of the phosphoprotein, (DSH; in
mammals DVL). The activated DSH inhibits GSK3, and prevents phosphorylation of CTNBB1, leading to the stabilization and accumulation of CTNBB1 within the cytoplasm. The CTNBB1 is then translocated to the nucleus, where it functions as a transcriptional co-activator, affecting transcription of target genes (Person et al., 2005a).

**Figure 8: Canonical WNT signaling pathway.**

This figure shows the fate of the canonical pathway both in the absence and the presence of WNT. In the absence of WNT, the GSK3 complex phosphorylates the beta-catenin (CTNBB1), which leads to proteolytic degradation of CTNBB1. In the presence of WNT, however, the beta-catenin is not phosphorylated and accumulates within the cytoplasm, ultimately translocating to nucleus and binding to transcription factor (TCF), resulting in the transcription of target genes (adapted from Person et al., 2005, Katoh and Katoh 2007, and Yokoyama et al., 2008).

**Non-Canonical Pathway**

In the non-canonical pathway, the WNT signal is understood to be mediated through FZD independently, without the involvement of the LRP5/6 co-receptors. The signal is then transduced to DVL and causes its activation.
The planar cell polarity cascade of the non-canonical pathway appears to be involved in valve development (Logan and Nusse, 2004). In this pathway, the activated DVL, activates dishevelled associated activator of morphogenesis 1 (DAAM1), which leads to: (1) activation of Rho GTPase, which results in the activation of Rho Kinase (ROCK) leading to actin polymerization through the activity of the actin binding protein profilin; (2) activation of Rac GTPase, which results in the activation of c-Jun terminal kinase (JNK), which in turn activates the transcription factor (AP-1) resulting in the transcription of target genes (Habas and Dawid, 2005) (Figure 9).

Figure 9: Non-canonical WNT signaling pathway.

In the Planar Cell Polarity (PCP) non-canonical pathway, the WNT-FZD complex activates the DVL and through DAAM1 mediates activation of RHOA and RAC which cause activation of ROCK and JNK subsequently. JNK1 is then translocated to the nucleus and binds to AP-1 causing transcription of target genes. In the WNT-Ca2+ pathway, the activated DVL causes release of intracellular calcium through G-proteins. The accumulated calcium results in the activation of calcium sensitive proteins, such as CAMKII, PRKC and CaN (PPP3R) Translocation of these proteins to the nucleus results in activation of NFKB and NFAT causing transcription of target genes (adapted from Habas and Dawid et al., 2005).

In the WNT/Ca2+ signal transduction cascade, the activated DVL protein causes release of intracellular calcium through trimeric G-proteins. The accumulation of
intracellular calcium activates the calcium sensitive proteins, protein kinase C (PRKC), calcium/calmodulin dependent kinase II (CAMK2/CaMK-II), and calcineurin (CaN alias PPP3R) (Figure 9). The activation of these proteins results in a variety of downstream effects including, regulation of transcription, tissue separation, inhibition of CTNBB1 to negatively regulate dorsal axis formation, and regulation of ventral cell fates (Komiya and Habas, 2008).

1.3.3.1 Role of WNT signaling pathway in valve development

WNT is expressed in various stages of valvulogenesis. Studies on murine models showed expression of: Wnt2, predominantly in mesenchyme (E12.5); Wnt4 and Wnt9b, co-expressed in endothelial cells (E12.5); Wnt3a and Wnt7b co-expressed in remodeling atrioventricular and semilunar valves (E17.5) (Yokoyama et al., 2007, van Amerongen and Nusse, 2009).

The expression of multiple WNT pathways genes in endocardial cushion mesenchyme and endothelial cells is suggestive that these WNT genes play a role in valvulogenesis. Endothelial cell-specific loss of Ctnbb1 in mice resulted in deficient development of endocardial cushions, demonstrating that the canonical Wnt signaling pathway is required for EMT during endocardial cushion formation (Alfieri et al., 2010). By manipulation of the Wnt receptor, Frzb, Person et al., 2005, demonstrated that Wnt signaling was responsible for promoting mesenchymal cell proliferation in the AV cushions. Further gene and cell specific knockdown studies have shown the role of Wnt2 in cardiac lineage differentiation in embryonic stem cells and Wnt9b in epicardial cell activation (Person et al., 2005a).
1.3.3.2 Role of Wnt signaling pathway in development of valve disease

Aortic valve calcification is an active process involving various mechanical and biochemical factors, leading to valve degeneration and stenosis. The valvular interstitial cell activation to osteoblasts and bone formation is one of the hallmarks of aortic valve calcification. Alfieri et al., 2010, studied the expression of mouse Wnt in remodeling valves and found expression of Wnt3a and Wnt7b ligands in the fibrosa surface of the valve leaflets after birth suggesting their role in valve stratification. Both ligands are known to play a role in osteoblast lineage commitment and development (Alfieri et al., 2010). The key element of the valve fibrosa layer is its highly organized collagen fibers and the ECM proteins. Studies on cultured aortic VIC, show that Wnt signaling is necessary for induction of genes responsible for ECM organization and structure (Logan and Nusse, 2004, van Amerongen and Nusse, 2009).

Albanese et al., 2017, conducted a study exploring the role of the Wnt signaling pathways in calcification by comparing the tissue distribution of WNT5A, WNT5B and WNT11 in non-calcified and calcified aortic valves, and human aortic valve interstitial cells (HAVICs). WNT5A was present in focal areas of calcification, whereas, WNT5B and WNT11 were seen in inflammatory cells and activated myofibroblasts in areas of calcified foci. Western blotting of stenosed valves and BAV showed abundant expression of all three types of WNT. Treatment of HAVICs with GSK3 inhibitor reduced the mineralization significantly (Albanese et al., 2017). These findings suggest a potential role for Wnt signaling pathways in calcification of valves.

1.3.4 Interplay of signaling pathways

Studies discussed in the above sections imply that there is an interplay between all three major signaling pathways. This is confirmed by requirement of both NOTCH,
and BMP2, a member of the TGF-beta family, for completion of EMT by maintaining SNAIL1 and 2 activity (Timmerman et al., 2004, Kovacic et al., 2012, Yu et al., 2015). Study of mouse models show Notch1 to play a role in regulation of Bmp and T-box transcription factor (Tbx2) through a negative feedback mechanism. Deficiency of Bmp2 and Tbx2 resulted in AVC defects (Ma et al., 2005, Harrelson et al., 2004). Targeted mutagenesis indicated that Bmp2 is a critical signal in EMT induction and cushion formation in the AVC (Ma et al., 2005), and Bmp2 is capable of independently specifying a field of cardiac progenitors as a heart valve-inducing region (Rivera-Feliciano and Tabin, 2006). Further comprehensive analysis of these signaling pathways may help in identification of common signaling modules and provide a greater insight into the genetic basis of valve development.

1.3.5 Gene Ontology

Gene Ontology (GO) is an international bioinformatics project with the goal of unifying the representation of genes and gene product attributes from the biological literature (Ashburner et al., 2000). This is achieved by translating data describing genes and gene products from primary literature using a controlled vocabulary. Their attributes are captured in terms of three distinct ontologies: ‘molecular function’, ‘biological process’ and ‘cellular components’ (Schuurman and Leszczynski, 2008, Gene Ontology et al., 2013). The biological attributes are organized in a hierarchical manner, based on their conceptual relationships, allowing specific attributes to be nested within broader concepts. The importance of an ontology with a controlled, yet enterprising, vocabulary was made evident when attempts were made to use common language in gene annotations. Standardization of vocabulary across taxa allows an authentic interpretation and distribution of the terms by any party, without erring due to the linguistic ambiguities and differences found across time within various
disciplines (Lovering, 2017). It also facilitates uniform queries across databases and provides a systematic tool for high-throughput data analysis (du Plessis et al., 2011).

To date, there have been over 40,000 unique biological vocabulary terms created and over 155,000 peer-reviewed scientific papers have been reviewed. Genes and their products are annotated with attributes identified by multiple or single source(s) of evidence, with the maximal number of most specific GO terms as appropriate. This results in a large analytical bioinformatics database which incorporates data from a wide array of sources.

1.3.5.1 Gene Ontology Overview

Each protein has its own unique reference ID, for human proteins this is acquired from UniProtKB. All annotations to that respective protein are then associated with its respective UniProt ID so as to avoid confusion and error. An annotation is made using a GO term which has its unique GO identification number (Figure 10). A GO term is also accompanied by a concise definition and a list of synonyms. The relationships between GO terms can be graphically represented in the form a directed acyclic graph (DAG), allowing more flexibility than a linear hierarchy, and allows for each term to have multiple relationships with more than one parent and child terms. A parent term represents a more general description of the attribute and its child terms, describe more specific subtypes. For example, the term, ‘semi-lunar valve development’ (GO:1905314) is a parent term of the term, ‘aortic valve development’ (GO:0003176), which in turn, has a child term, ‘aortic valve morphogenesis’ (GO:0003180). Figure 10 below shows a part of the ancestor chart of these terms. Relations between terms are represented by lines with arrows pointing towards the parent term. The relations existing between parent and child terms can take multiple forms depending upon the
conceptual interaction between them, including, ‘is a’, ‘part of’, ‘regulates’, ‘occurs in’ amongst others. In QuickGO each relation type is assigned a specific color (Figure 11).

Figure 10: Snapshot from the QuickGO interface.

The QuickGO interface provides information such as the GO identification number, GO term and the definition, as well as a graphical view and all the annotations associated with the terms. Information obtained from this interface can be used to create new annotations.

Figure 11: A portion of a Directed Acyclic Graph (DAG).
The DAG represents the hierarchical relationships of the GO term ‘aortic valve development’. Above this term, is its parent term, ‘semi-lunar valve development’, whereas, beneath it, is its child term ‘aortic valve morphogenesis’. A search for parent terms will retrieve annotations from the database, not only associated with the parent term itself but to the child terms as well. Relations between terms are represented by lines with arrows pointing towards the parent term and are assigned a specific color. In this case the blue line from aortic valve morphogenesis indicates that it is a ‘part of’ aortic valve development, and its nature ‘is a’ heart valve morphogenesis. These nested relationships continue back until the term ‘biological process’ as the root term which is the parent to all these terms (adapted from https://www.ebi.ac.uk/QuickGO/).

1.3.5.2 GO consortium

The GO consortium is an international collaboration of research groups and participating databases of GO. It is responsible for reviewing, updating and expanding the ontology and annotation files, based on biological knowledge and the needs of its users. Editors within the consortium are responsible for the development, and maintenance of the vocabulary and hierarchy of the ontology. Whereas, the biocurators are responsible for creating new annotations for inclusion into the database. Any dispute or request for complex new terms are resolved via the GitHub database. The creation of new vocabulary for GO is often done preemptively by editors, in discussions with experts in the field to define a conceptual space within which expected future annotations will exist (Gene Ontology et al., 2013). Any gaps in the ontology are later filled by editors and biocurators.

Annotations from this project are assigned the source, ‘BHF-UCL’, as they contribute to the functional gene annotation team at UCL funded by the British Heart Foundation, which focuses on annotations relevant to cardiovascular development and pathology (Khodiyar et al., 2011, Khodiyar et al., 2013, Lovering et al., 2018).
1.3.5.3 Curation

Manual Curation

Manual Curation of GO is carried out by biocurators, trained to read and extract information based on published literature. Albeit not perfect and subject to human error, manual curation remains the gold standard of annotations, as the human ability to interpret and designate scientific data is still superior to artificial methods. The annotation provides a comprehensive summary of a given biological entity, including, interpretation of the experimental results based on appropriate evidence code, and the GO terms describing its molecular function, biological process and subcellular address (Balakrishnan et al., 2013).

There are two approaches of carrying out manual curation, protein centric and process centric. Protein centric focuses on the annotation of an assigned protein or family of proteins, however, it has the disadvantage of not viewing the protein and its roles in a larger perspective. The process centric approach, on the other hand, involves biocurators annotating all proteins involved in a single process, e.g. aortic valve development (Lovering et al., 2018). Both methods have contributed to GO annotation datasets. While the protein centric approach allows comprehensive representation of the functions of a gene product, the process-centric approach helps in pathway identification.

Electronic Curation

Electronic annotations are commonly used within GO and are based on methods that apply a set of manual annotations to all proteins with a specific domain or based in orthology predictions. The protein domain or signature associated electronic annotations are less specific because the annotations have to be correctly applied to all
proteins with the defined domain. Nevertheless, this method has the advantage of being cheaper and less time consuming than manual curation, based on annotation of single gene products (Huntley et al., 2015).

Electronic orthology-based annotations are created using the protein orthology prediction tool, such as Ensembl Compara. This tool provides cross-species resources and analyses, at both the sequence level and the gene level, allowing comparison to one another in order to produce gene trees, infer homologues and produce gene families. Gene Trees allow the inference of pairwise relationships between and within species. Genes in different species and related by a speciation event are defined as orthologues, whereas, genes belonging to the same species and related by a duplication event are defined as paralogues (Huntley et al., 2015).

Ensembl Compara identifies 1:1 orthologues, 1: many orthologues and many: many orthologues. The electronic pipeline runs each month at EMBL-EBI which transfers existing GO annotations supported by experimental evidence across to 1:1 orthologues only, although the human proteome only receives annotations from mouse and rat through the Ensembl Compara pipeline.

1.3.5.4 Aortic Valve Development in Gene Ontology

As discussed previously, studying aortic valve development at both morphological and genetic levels is crucial to obtain a multifaceted understanding of the subject matter in order to advance treatment of aortic valve disease. The aortic valve related GO annotation files were still lacking sufficient description of this process. This project takes a bioinformatic approach within the ontological framework of the GO database to functionally annotate gene products related to aortic valve development using experimental evidence from published literature.
Due to logistical and ethical concerns, experimental literature on human genes is quite limited, and, therefore, the relevant experimental literature is generally based on murine models due to their genetic amenability and evolutionary conservation compared to humans. The majority of Next-generation Sequencing (NGS) analysis tools, hence, currently use mouse phenotype data (or, the Genomics England PanelApp data from the 100,000-genome project). However, it is important to note that targeted knockouts of these mouse genes may, either result in embryonic lethality, or, not exhibit any abnormal valve phenotype due to the presence of paralogs or other genes which can compensate for the knocked-out gene.

The annotation of proteins involved in valve development will, therefore, enable identification of other proteins within the same signaling pathway that may be working in a polygenic manner to cause the valvar defects. This will eventually provide a resource for NGS analysis of patients with BAV, other AVDs, or other valve diseases. By targeted sequencing of these genes in a patient, or by using these annotations to filter the identified patient’s variants to identify more likely candidate genes contributing to disease.
1.4 **Congenital Aortic Valve Defects**

Congenital heart defects (CHD) are the most common birth defect affecting 6-8 per 1000 live births, but including bicuspid aortic valve disease (which is generally considered as a separate entity) this figure rises to 2 – 3% of live births, accounting for almost 200,000 deaths annually worldwide (Global Burden of Disease Study 2013 Collaborators, 2015). One of the ways of classifying CHDs is based the presence of cyanosis: cyanotic and acyanotic diseases (Rohit and Shrivastava, 2018). Aortic valve disease (AVD) is a type of acyanotic CHD.

Defects leading to AVD could either be due to fusion of aortic valve leaflets, i.e. unileaflet (commonly referred to as unicuspid) or bi-leaflet valves with or without dysplasia; dysplasia of valve leaflets alone without fusion of commissures (Kim et al., 2014, Price and El Khoury, 2012); or obstructive aortic lesions above or below the valve itself (supravalvar/subvalvar aortic stenosis). This could result in obstruction to or leakage and backflow of blood ejected from the LV into the ascending aorta, back into the left ventricle, providing a significant substrate for prenatal, neonatal and adult mortality and morbidity throughout the life-course from fetal life to adulthood (Gilboa et al., 2010, Bouma and Mulder, 2017, Hameed and Sklansky, 2007).

1.4.1 **Extracardiac impacts of Congenital AVD**

In recent decades, advances in the diagnosis and management of CHD have greatly improved, increasing the longevity and quality of life of patients, with upwards of 90% of affected children now surviving to adulthood (Gurvitz et al., 2016, Karsenty et al., 2015). Nonetheless, the cardiac and extracardiac impacts of CHD is seen in patients even after treatment of the primary lesion, posing great challenges to surgeons, other
medical professionals, and patients themselves; thus, morbidity is now a focal point for current research.

Studies have shown that in complex CHD, up to 50% of infants suffer from some type of neurodevelopmental impairment (Marino et al., 2012, Sanz et al., 2017). Children born with AVD, have an increased incidence of executive dysfunction, especially in working memory and behavioral flexibility, which is more commonly seen in males (Sanz et al., 2017, Laraja et al., 2017). While the exact mechanism responsible for such abnormal neurodevelopment is not yet known, two likely theories exist. The first suggests that the brain develops differently due to presence of intrinsic (epi) genetic factors in CHD. Since there are shared genetic pathways responsible for the development of heart and brain, a defect in one of these shared genetic pathways could lead to abnormal development of both the brain and the heart (Marino et al., 2012).

The second theory suggests that circulatory alterations may arise owing to presence of a CHD, such as AVD, and that this might lead to a consequent decrease in oxygen and nutrient supply to the brain resulting in defective cerebral development (Mebius et al., 2017, Hutter et al., 2010). These hemodynamic alterations could be due to a decrease in oxygen level within the blood reaching the brain, or decreased volume of blood and, therefore, insufficient oxygen delivery to the cerebral circulation, or both. Whether one hemodynamic factor or a combination of both is responsible for the poor neurodevelopmental outcomes, the relation of neurodevelopmental abnormalities to CHD suggests the need for the early repair of the cardiac defect to minimize the detrimental effect on the neurodevelopment of the patient. Furthermore, studies looking into the neurodevelopmental outcomes in patients who have undergone cardiac surgery suggest that risk factors during the perioperative period and cardiopulmonary bypass have not had a strong impact on neurodevelopmental
outcome as expected, rather, post-operative factors such as need for extracorporeal membrane oxygenation or assistant device support have a significant influence on the neurodevelopmental outcomes (Gaynor et al., 2015, Hovels-Gurich, 2016, International Cardiac Collaborative on Neurodevelopment, 2016).

Other extracardiac manifestations of congenital AVD seen in adulthood, include intracranial aneurysm, anemia and renal dysfunction, amongst others. The significant effects that these extracardiac abnormalities have in patients with congenital AVD, highlights the need for further research in understanding the development of AVD, both structurally (microscopic and macroscopic levels) and genetically (Collins et al., 2008, Schievink et al., 2010, Gaeta et al., 2016, Wernovsky and Licht, 2016, Mebius et al., 2017).

### 1.4.2 Congenital Aortic Stenosis

Aortic Stenosis (AS) commonly presents at a later stage of adulthood and is, generally, a result of the malformation of the leaflets of the aortic valves and is often seen with bi-leaflet aortic valves. Bi-leaflet (or commonly known as bicuspid) aortic valve (BAV) is the most common congenital heart defect affecting about 0.5 - 2% of the population (Braverman et al., 2005), and can cause rapidly progressive AS in infancy and childhood (Pomerance, 1972). BAV is also the commonest cause of AS in patients above 60 years of age and usually presents in persons older than 20 years. Patients suffering from AS secondary to BAV often require aortic valve replacement 5 years prior to patients with a tri-leaflet valve (Mautner et al., 1993).

AS accounts for 5-6% CHD and its presentation in neonatal patients is often critical requiring emergency ballooning. This is due to the severe obstruction frequently caused by presence of a uni-leaflet aortic valve, instead of the normal tri-leaflet aortic
valve. Although, uni-leaflet (or commonly known as unicuspid) aortic valves (UAV) in theory and practically, cause more obstruction, it has been less commonly reported in aortic stenosis in adults. Whether, this is because a lesser proportion of patients with UAV survive to adulthood requires further research.

A typical clinical picture of a neonate with aortic valvar stenosis is illustrated by the following published case report (Manvi et al., 2014):

A 23-day-old neonate was born to a primigravida mother by full term normal vaginal delivery. The neonate was apparently asymptomatic and remained well until 15 days of life following which he developed hurried breathing, difficulty in feeding and excessive perspiration. On examination, the patient had heart rate of 150/min, respiratory rate of 60/min and a palpable liver. Auscultation revealed a grade 3/6 ejection systolic murmur present in the aortic area. Chest X-Ray showed cardiomegaly. Two-dimensional echocardiography showed bicuspid aortic valve with critical (severe) valvar aortic stenosis. There was a severe left ventricular systolic dysfunction with ejection fraction of 25%. Left atrium and left ventricle were dilated. Aortic valve annulus was 8 mm. The patient was taken up for emergency balloon aortic valvotomy. Post procedure echocardiography showed well-opened aortic valve with gradient of 20 mmHg with a significant improvement in the left ventricular systolic function. Ejection fraction was 55%. There were no local or systemic complications noted. On follow up a month post discharge the child had significant clinical improvement on physical examination with echocardiography showing well-opened aortic valve and normal left ventricular ejection fraction (60%).

AS presenting in the neonatal period is a unique entity with pathology, clinical presentation and outcome different to left ventricular outflow tract lesions presenting
in later life. Although, congenital AS is uncommon, representing 3-5% of all patients suffering from congenital heart disease, it contributes to two thirds of obstructive lesions affecting the left ventricular outflow tract (Hraška, 2016).

Patients suffering from neonatal AS often present with symptoms of heart failure and appropriate and timely management is, therefore, essential. Management of neonatal AS is quite challenging due to different morphology of the valve in neonates, hypoplasia of the aortic root, association with abnormalities of other left heart structures, and the presence of left ventricular systolic dysfunction. Morphological examination of the valves in neonatal AS, reveals only one commissure extending to the sinotubular junction whereas the other two commissures are represented by folds or raphe and the leaflet suspended below the level of the sinotubular junction. In order to treat neonatal AS, expansion of the narrowed aortic orifice needs is often necessary (Leung et al., 1991, Paladini et al., 2002).

Hastrieter and colleagues divide the pathologic entities presenting as aortic valve stenosis into three separate groups. The first, aortic stenosis as an isolated lesion, is characterised by a relatively well-formed valve often bicuspid with an adequate aortic annulus, and an ascending aorta without cardiovascular malformations. This doesn’t typically present in neonatal period. Second are, a group of patients whose lesion may be considered to represent primarily contracted endocardial fibro-elastosis with only secondary involvement in aortic valve. And third, a group characterised by marked deformity of the aortic valve with thickening, poor mobility and often a uni-leaflet or perforate membrane structure. In this group, hypoplasia of the aortic root and the ascending aorta, and coarctation of the aorta are often seen, as are associated abnormalities such as mitral stenosis and regurgitation, and secondary endocardial fibro-elastosis. This latter group is the most common one to present under one month
of age (Hastreiter et al., 1963). From a surgical point of view, the latter group is often classified according to size of the ventricle, either, dilated or normal sized, or, a small left ventricle, leading to the fundamental question, “how small is too small?”. However, presence of a severe obstruction unamenable to surgical intervention either inlet or outlet, makes the left ventricular size somewhat less important.

Interventional outcomes have improved in recent years as a consequence of better patient selection, perioperative management and advances in catheter technology. Further research and development in the understanding of the aortic valve morphology, imaging and transcutaneous approach will provide better definition of valve morphology and aid in patient selection for surgical versus catheter-based intervention. This will allow significant modification of the natural history of this disorder, including fetal intervention for the salvage of the hypoplastic left ventricle.
1.5 **Detailed structure of the aortic valve in health and disease**

The heart is a vital organ that should be studied as a whole, keeping in view its structure and function (Zimmerman, 1966). It is often, due to a unilateral method of studying the heart, that some surgeons and morphologists often find themselves in a mêlée over what the most appropriate terminology is, to describe certain anatomical structures within the aortic root. Correct morphological study of the aortic root and valve is, therefore, best done by examining the aortic root as a functional valvar complex. This complex can then be analysed at three levels: sinutubular junction, sinusal, and basal.

1.5.1 **Normal Aortic Root and Valve**

The *aortic root* is defined as the part of the ventricular outflow tract that extends from the base of the aortic sinuses to the level of the sinotubular junction. It consists of the base of the aortic valve leaflets, the leaflets, the commissures, the inter leaflet triangles, the sinuses of Valsalva, and the sinotubular junction (Figure 12). Contained within two of the three sinuses are the origins of the coronary arteries. Each component of the aortic root has an optimal gross and microscopic structure, all of which contribute to the function of the aortic root as a whole: intermittent, unidirectional, transmission of large volumes of blood whilst maintaining, laminar flow, minimal resistance and the least possible tissue stress and damage, during fluctuating hemodynamic conditions. The effective functioning of the aortic root is critical in maintaining optimal coronary flow, left ventricular performance and preservation of the healthy myocardium (Piazza et al., 2008, Ho, 2009).

The sinutubular junction is a ‘ring’ like structure, composed of thickening of the aortic wall, that forms the distal boundary of the aortic root. It supports the zeniths of the peripheral attachments of each *valvar leaflet*, a ‘unit’ of tissue separating the
ventricular and arterial sides of the root. Normally, the aortic valve is composed of three leaflets, although the dimensions of each may vary from one another (Roberts, 1973, Ho, 2009). The leaflets are arranged in a semilunar fashion with three half-moons present within a normal valve, which, if cut open and laid flat, would look somewhat like the ‘golden gate bridge’ (Figure 12). When in their closed position, during diastole, the leaflets co-apt along their free edges overlapping each other in a zone of apposition or commissure, since the cross-sectional area of the aortic root is less than the sum of the areas of the leaflets. According to conventional literature, the point of junction between the zenith of the peripheral attachment of each leaflet to the sinutubular junction of the aortic root is termed as the commissural end, whereas, the base is formed when the by the nadirs of the leaflet attachments. The aortic valve leaflets form the hemodynamic junction between the left ventricle and the aorta (Figure 12). All the structures distal to the hemodynamic junction are subject to arterial pressure, whereas all the structures proximal to the junction are subject to ventricular hemodynamics (Sutton III et al., 1995, Becker, 1996, Tretter et al., 2016).

Below the sinutubular junction, three extensions of the aortic root are formed within the arterial wall, called the aortic sinuses of Valsalva. These sinuses are confined proximally, by the attachments of the valvar leaflets and distally, by the sinutubular junction (Figure 12). Two of the three sinuses give rise to the coronary arteries and are thus, named accordingly (left, right and non-coronary). Identification of sinuses allows identification of leaflets, which is important since it is used to help determine site of fusion in uni-/bi-leaflet valves (Anderson et al., 1991, Tretter et al., 2016). The sinus wall, albeit predominantly made up of aortic wall, is thinner than the aortic trunk, and expand down to the base of the aortic root where the circumference narrows slightly (Sutton III et al., 1995). Although the precise function of the sinuses of Valsalva is
unclear, dimensional studies on aortic root during each cycle has shown that the sinuses account for significant variations during opening and closing of the valve (Angelini et al., 1989). Furthermore, there is evidence that the vortices created in the sinuses lead to reduced stress on the aortic valve leaflets and support coronary blood flow during diastole (Leyh et al., 1999, Charitos and Sievers, 2013).

Figure 12: Detailed drawing of the aortic valve.

The aortic valve is made of three semi-lunar leaflets, the left coronary leaflet (LL), cut in the figure above, the right coronary leaflet (RL) and the non-coronary leaflet (NCL). Between these leaflets are spaces called interleaflet triangles (highlighted in red lines), extending from the tip of the sinutubular (ST) junction to the ventriculo-aortic (VA) junction. Note that in presence of aortic valvar stenosis, where there is fusion between leaflets, there is a reduction in the height of the interleaflet triangles (white double-edged arrows), making them ‘annular’ in shape as opposed to the normal ‘semilunar arrangement’. The membranous septum (MS) and the mitral valve (MV) are also shown in this picture (Adapted from Gemma Price medical illustrations).

The base of the aortic root is defined by the nadirs of the attachment of the leaflets. Although, the base sits on partially fibrous (and partially muscular) support, it is important to note that the base of the valve is not fixed, but rather, expands and shrinks during ventricular diastole and systole, respectively (Sutton III et al., 1995). This site, often incorrectly referred to as the aortic ‘annulus’, approximates the region where the
surgeon sews a prosthetic valve. The lack of a continuous collagenous circle between the nadirs, is due to the interruption by the semilunar attachments, and therefore, calling it an annulus (i.e. ring) is not correct. On longitudinal sectioning of the aorta, one can appreciate that there is only one definitive “annular” (or circlet) structure which is the junction of the aorta with the ventricular structures, the ventriculo-arterial junction, although the sinutubular junction is also partially ring-like. As such the root of the aorta is not an annulus, but rather, shaped as a ‘tricorn’ due to the helical shape of the valve leaflets (Anderson et al., 1991) (Figure 13). The reason why the correct use of the term annulus is important, is not merely a difference in etymological choice, but rather, important to differentiate the semi lunar arrangement of the leaflets in normal valves from the annular (circular) attachment of leaflets in malformed hearts. This difference in appearance is seen due to the loss/reduction in height and width of the *interleaflet triangles* in malformed hearts (McKay et al., 1992, Tretter et al., 2016).

Figure 13: A diagram showing the points of attachment of leaflets of the aortic valve.
The lowest point of attachment of the leaflets at their base are referred to as Hinge Points. Note the virtual ‘ring’ that is commonly referred to as the aortic annulus is different to the actual shape of the aortic root, which is a tricorn, highlighted in pink. Above the hinge point plane is the anatomic ventriculoaortic junction where the ventricle meets the aorta. This point has been referred to by some authors as the base of the interleaflet triangle. However, there is some confusion within the literature as to what constitutes the base of the interleaflet triangle, with some referring to as the ‘hinge point plan’ as the base of the triangle (adapted from Frank Netter Medical Illustrations, Kasel et al., 2013, Tilea et al., 2013).

Underneath the apex of each commissure, thin layers of fibrous tissue separate the ventricle from the pericardial space. These tissue layers are called ‘interleaflet triangles’ and are responsible for the ventricular nature of the base of the valve. The boundaries of the interleaflet triangle have been described by Sutton III as; base formed by the anatomic ventriculo-aortic junction, and sides formed by the hemodynamic ventriculo-aortic junction with the height extending up to the apex of the commissure (Sutton III et al., 1995). The anatomical junction made between the ventricle and the arterial trunk takes the form of a circle over which the fibrous wall of the trunk is attached to the supporting ventricular structures by collagen. Whereas, the hemodynamic junction, in contrast, is marked by the semilunar locus of attachment of the leaflets. There is a disparity found between the morphological description of the interleaflet triangles and the functionality of the interleaflet triangles seen on echocardiography, which shows that the entire interleaflet triangle (to the level of the sinutubular junction) expands as the aortic root dilates. This indicates that the correct measurements of the interleaflet triangle are necessary to be able to understand the functionality of the interleaflet triangle (Sutton III et al., 1995, Tretter et al., 2016).

The triangle between the right- and non-coronary sinuses faces the right atrium. It is in direct continuity with the membranous septum proximally which contains at its base the bundle of His. This area is of special importance during aortic valve procedures, as injury here can lead to temporary or permanent conduction abnormalities, which may
require the implantation of a permanent pacemaker (Khawaja et al., 2011). Under the left and non-coronary triangle, the aorto-mitral curtain leads to the anterior mitral valve leaflet (Maizza et al., 1993). The triangle between the left and right coronary sinuses lies immediately behind the right ventricular outlet and is related to the space between the aorta and pulmonary trunk or infundibulum (Ho, 2009, Sutton III et al., 1995).

1.5.2 Abnormal Aortic Valve

Understanding the morphology of the abnormal aortic valve is essential for the improvement of surgical approach to reconstructing structures of the aortic valvar complex within a congenitally malformed aortic valve. The congenital anatomical abnormalities of the aortic valve are manifested as either, a uni-leaflet, or a bi-leaflet valve. The choice of using the terms ‘uni-leaflet’ and ‘bi-leaflet’ in this thesis, instead of the conventional terms, ‘unicuspid’ and ‘bicuspid’, respectively, is due to the term ‘leaflet’ being, etymologically, more appropriate when used to describe the portions of tissue of the aortic valve, rather than the term ‘cusp’, which linguistically means, a point of transition, or the point where two curves meet, and in anatomy, is used to describe a cone shaped prominence at the back surface of the tooth.

The familiar variant, out of the two, found in literature is the bi-leaflet aortic valve (BAV); this is most likely due to a higher incidence of BAV presenting clinically, later on in life, and as some authors include uni-leaflet aortic valve cases (UAV) as a variant of BAV.

**Bi-leaflet Aortic Valve**

BAV is one form of outflow tract abnormality in a spectrum of diseases of the left heart ranging from aortic stenosis to hypo-plastic left heart syndrome to coarctation of the aorta amongst others. It is characterised by two leaflets of the aortic valve rather
than the normal three, due to abnormal fusion of two valve leaflets to form one (Sans-Coma et al., 1996).

Although the exact mechanism causing development of BAV is unclear, there are different theories on the etiology of BAV. Some have suggested that a molecular abnormality in the extracellular matrix might cause development of BAV, as matrix proteins help in cell differentiation and leaflet formation during valvulogenesis (Eisenberg and Markwald, 1995), whereas, others provide evidence that BAV is associated with a defect in the NOTCH1 signaling pathway (Garg et al., 2005). Further studies looking at the effects of ECM genes and the NOTCH1 signaling pathway may lead to a better understanding of the mechanism of the development of BAV.

**Gross Anatomy of UAV/BAV valves**

The BAV has two commissures, with well-formed interleaflet fibrous triangles, with (if present) a third triangle that is vestigial and related to the raphe of the conjoined leaflets (Angelini et al., 1989, Duran et al., 1995, Anderson et al., 2009). The zones of apposition extend at both ends to reach the sinutubular junction, which, if looked from above, resembles the top end of a ‘fedora’. The uni-leaflet valve, however, has a zone of apposition that extends from the sinutubular junction to the centroid of the valvar orifice which is usually eccentrically positioned within the aortic root (Figure 14). This extension can be attributed to a single well-formed interleaflet triangle between the non-fused leaflets, with two vestigial interleaflet triangles. In both forms of valves, the lack of well-developed interleaflet triangles, be it one or two, results in the persisting leaflet tissue acquiring a more circular shape giving the base of the aortic root a more ‘annular (or ring like) shape as opposed to the ‘parabolic’ shape of the normal aortic root (Angelini et al., 1989, Anderson et al., 1991).
Figure 14: A visual representation of the appearance of unileaflet and bileaflet aortic valves.

The red dotted lines represent ridges of tissue called raphe that are found along the sites of fusion of the leaflets. Note, this representation of a bileaflet aortic valve (BAV) is not what literature refers to as a true BAV, as there is a presence of raphe indicating fusion of two leaflets to form one leaflet, in addition to a non-fused leaflet. According to the common Sievers classification, a true BAV does not have a raphe and instead, the valve has two unfused leaflets as opposed to three in the normal. Note how the top of the leaflet resembles the top of a fedora.

Along the sites of fusion of leaflets, there are ridges of tissue called raphe. Depending on the number of sites and nature of fusion of the leaflets, the valve has either one or two raphae (Sutton III et al., 1995) (Figure 13). Meticulous attention to the raphe is important whilst assessing the morphology of the aortic valve, since, due to the vestigial nature of the interleaflet triangle(s), clinicians, and morphologists alike, may mistake the uni-leaflet valve for a bi-leaflet valve. This is because it is difficult to assess the height of the zones of apposition with the raphae commonly being mistaken for leaflets zones of apposition, when the leaflets close during diastole (Disha et al., 2018). Personal communication with congenital cardiac surgeons has revealed that there’s often a discrepancy between pre surgical diagnosis of the nature of fusion and the findings on surgery. A better understanding of the anatomic nature of these raphae may help with correct diagnostic assessment and management of such cases.
Due to the increased incidence of bi-leaflet valves, most studies reported have looked into heart specimens with bi-leaflet valves (Angelini et al., 1989, Anderson et al., 1991, Duran et al., 1995, Sabet et al., 1999). However, a detailed morphometric study looking into critical aortic stenosis in infants, analysed the morphology of uni-leaflet valves (McKay et al., 1992), as uni-leaflet valves are, usually, the cause of critical aortic stenosis in infants. In bi-leaflet valves, Angelini et al. found 84% to have a raphe present, 64% of the specimens to have fusion between the left and the right leaflets, and 78% of adult samples had calcification confined to the leaflets. Duran et al. found a similar trend whilst assessing the sites of fusion of leaflets. The team further studied the morphology of leaflets amongst the specimens; 40% of the specimens were stenotic due to calcification whereas 25% showed focal signs of calcification, 10% of the specimens had infective endocarditis, and the remaining had no complications. A similar trend was seen by McKay et al. when analysing the fusion of leaflets in uni-leaflet valves. In 95% of the samples, a patent commissure was found between the non-coronary and left coronary sinuses, indicating fusion of the other two leaflets. The height and the base of the interleaflet triangles beneath either patent or imperforate commissures were both reduced as compared to the positive controls. Measurements of the circumference of the aorta at the aortic root was also shown to be approximately half of the normal values. Furthermore, the length of the free edge was reduced for each leaflet. Due to such an abnormality, the expansion of the aortic root is restricted, and prevents sufficient opening of the valve. This left ventricular outflow tract obstruction creates a hemodynamic abnormality, which subjects the leaflet to increased stress and vibration (Bellhouse and Bellhouse, 1969, Thubrikar et al., 1980). This is thought to cause the leaflet to thicken and develop nodular outgrowths on its
luminal aspect. McKay et al. showed at least one leaflet in each stenotic valve had nodularity, and majority of all the leaflets had a mixture of small and large nodules.

Calcium deposition and fibrosis of the BAV progresses with age and is predominantly confined to the raphe and the base of the leaflet. Although, the calcification process occurring in BAV may be similar in terms of cellular and molecular mechanisms, to that seen in a tricuspid aortic valve, the process of calcification is reported to be accelerated (Mathieu et al., 2015, Lindman et al., 2016).

Imaging studies have shown several compensatory mechanisms in BAVs, such as significant folding of the valve tissue, and increased doming of the valvular leaflets during the cardiac cycle. Due to such compensatory mechanisms along with other occurring processes, most patients require surgery, valve repair or replacement during the course of their lifetime (Pomerance, 1972, Lewin and Otto, 2005).

Since the presentation of bi-leaflet valves occurs across a spectrum of ages, and patients with uni-leaflet valves often die in infancy, it is hard to pinpoint the cause of the malformation of the aortic valve. This gives rise to the debate - the answer to which is unknown - whether the malformed aortic valve developed from the start as uni- or bi-leaflet; or whether the leaflets fused later on during fetal development; or if they fused during later life due to calcification and other overlapping mechanisms. Whereas, some authors, (Roberts, 1973, Leung et al., 1991) have suggested that the circumference of leaflets can be helpful in making the distinction, others (Angelini et al., 1989) stressed the importance of the number of sinuses present, since, during heart development, the sinuses act as the structure that supports the leaflets and develop from the same primordia.
Valve leaflet orientation and morphology can vary; however, studies have shown fusion between the right and the left leaflet to be most common phenotype, occurring in 86% of the samples studied (Toufan Tabrizi et al., 2018, van Engelen et al., 2014, Murphy et al., 2017). Almost all specimens studied showed three sinuses with a bi-leaflet valve, which indicated that the valve initially developed with three leaflets, but subsequently, fusion occurred between leaflets, resulting in a bi-leaflet valve (Angelini et al., 1989, Duran et al., 1995). Animal models have been used to evaluate the cause of the fusion. The models, reported thus far, predominantly show fusion of the right and left valvular cushions at a very early stage of valvulogenesis, and not from absence of valvar cushions or lesions acquired after normal valvulogenesis (Sans-Coma et al., 1996).

Despite different models, the reason for fusion is not yet clear (Fernández et al., 1998, Fernández et al., 2000). Some studies have attributed etiology of the BAV to certain genetic factors (Emanuel et al., 1978, Sans-Coma et al., 1996) or altered fetal hemodynamics causing aortic valve maldevelopment (Moore et al., 1980, Clark, 1996). Moore et al. states that the relative disproportional blood flow through the two outflow tracts, with reduced flow in the aorta could favor fusion of the commissures of the aortic valve after normal valvulogenesis. Recent studies show that this may be due to the presence of cytokines and adhesion molecules in the aortic valve leaflets via a TGFB1 dependent pathway in response to the altered hemodynamic state (Dvorin et al., 2003, Latif et al., 2005, Sucosky et al., 2009). These findings lead to another key question; whether congenitally malformed valves are the cause or the result of the often-associated left ventricular disease? In other words, is there an overlap in etiology between LV cardiomyopathy and AVD as has been previously suggested during fetal life (Sharland et al., 1991)?
1.5.3 The Myocardium in AVD

1.5.3.1 Left Ventricular Remodeling

Left ventricular hypertrophy (LVH) is a remodeling response seen when there is increased pressure exerted on the left ventricle (LV). Although, seen as a physiological response with long-term exercise in athletes, this can also be elicited as a result of various pathologies, such as, aortic stenosis (Donaldson, 1982).

Due to the obstruction of the aortic valve (in aortic stenosis) or incompetence (in aortic regurgitation), there is pressure and volume overload on the LV leading invariably to life threatening conditions, such as cardiac failure (Rader et al., 2015). Timely aortic valve repair/replacement are effective in relieving the hemodynamic burden and improves the symptoms and survival of the patients. However, since intervention isn’t considered until presence of symptoms such as cardiovascular collapse or acidosis in neonates, or syncope or angina, in adults because they indicate a poor prognosis, and the majority (two-thirds) of adult patients do not develop these symptoms at rest, the timeline of progression of the disease does not often correlate with the clinical presentation (Marquis-Gravel et al., 2016, Baumgartner et al., 2017, Kanwar et al., 2018). This results in a delayed intervention which may prevent regression of left ventricular hypertrophy (Nepper-Christensen et al., 2017, Everett et al., 2018). Furthermore, according to current guidelines laid out by the American College of Cardiology/American Heart Association, left ventricular hypertrophy, even if severe, is not an indication for aortic valve intervention (Nishimura et al., 2014). Similarly, in babies born with critical aortic stenosis, due to the pressure overload throughout fetal life, the LV often undergoes compensatory changes. Therefore, even if an intervention is carried out, the damage may be irreversible (Friedman et al., 2011, Olasinska-Wisniewska et al., 2013, Friedman et al., 2015). Studies show that incomplete
regression of left ventricular hypertrophy is associated with poor early post op and worse long-term outcomes (Tzikas et al., 2011, Stulak et al., 2011, Lindman et al., 2014).

Although, generally perceived as a compensatory mechanism, studies on humans and animals show that presence of left ventricular hypertrophy (LVH) does not improve the functioning of the heart, nor does its absence worsen cardiac function, but in fact, its presence increases the incidence of LV systolic dysfunction and heart failure (Kupari et al., 2005, Jayaprasad, 2016). This suggests that the development of hypertrophy, may in fact be an epiphenomenon to pressure overload, that contributes, rather than prevents, left ventricular dysfunction. The two major ‘compensatory’ processes seen in response to chronic LV overload and rising intra-cavity pressure in AS are, concentric hypertrophy of the left ventricle and diffuse myocardial fibrosis (Shah et al., 2014, Schirone et al., 2017). Patients with BAV-stenosis show significantly larger left ventricular volumes and left ventricular outflow tract diameters in comparison to those with a tricuspid/tri-leaflet aortic valve (TAV), which explains why there is higher incidence of LVH found in the former (Disha et al., 2017, Kong et al., 2017). The increase in myocardial muscle mass and alteration of the LV radius and wall thickness is seen as an attempt to maintain normal wall stress, but contrary to normalizing the cardiac functioning, it is sometimes viewed as a slow poison (Byrne et al., 2016).

A morphological study of cardiac specimens with critical aortic stenosis in infancy was carried out in context of echocardiography obtained from live patients within the same age group (Leung et al., 1991). Morphologically, the study showed a spectrum of ventricular abnormalities. On one end of the spectrum, the samples had a small left ventricular cavity, thick postero-apical walls, and a small mitral orifice with
hypoplastic and thickened papillary muscles, whereas, on the other end of the spectrum, samples exhibited a dilated left ventricular cavity with thickened walls and endocardial fibro-elastosis. Leung et al., also showed significant differences in the echocardiographic dimensions of the left ventricular cavity, and the orifices of its inflow and outflow between survivors and non-survivors after undergoing open valvotomy to relieve AS. This indicated that unfavorable cardiac anatomy of the non-survivors was indicative of low cardiac output and heart failure.

1.5.3.2 Effect of Aortic Valve Intervention on LVH

While, aortic valve intervention relieves pressure overload, the presence of concentric hypertrophy (increased relative wall thickness) is regarded as an important predictor of post-operative morbidity and mortality (both early and delayed). The regression of hypertrophy postoperatively depends on the severity of LVH prior to valve intervention, and the nature of the valve (Krayenbuehl et al., 1989, Lindman et al., 2014, Rader et al., 2015). Studies have shown marked regression of LVH in patients who had a (stent-less) bio-prosthetic valve than patients who had a mechanical valve, signifying the need for development of durable bio-prosthetic valves (Jin et al., 1996, Maselli et al., 1999, Dunning et al., 2007). Nevertheless, it is important to realize that studies show approximately only a mere 30% of the patients show a decrease in LV thickness postoperatively (Lim et al., 2008, Repossini et al., 2012, Beach et al., 2014). This suggests that awaiting the development of symptoms as an indication for intervention results in irreversible changes in LV remodeling and, therefore, a missed opportunity to intervene when such remodeling is reversible, greatly affecting the survival of the patient.
In some cases of AS, however, LVH does not regress despite aortic valve intervention, due to the presence of a concomitant disease that mimics LVH. One such disease commonly seen in adults is cardiac amyloidosis (Rind et al., 2018). Recent studies have shown that 6% and 13% of patients with AS over the age of 75 undergoing surgical aortic valve replacement and those referred for trans-catheter aortic valve replacement (TAVR), respectively, have occult cardiac amyloidosis (Treibel et al., 2016, Scully et al., 2018). Prognosis in such patients is often poor and treatment is dependent on the extent of cardiac involvement. Presence of transthyretin cardiac amyloid in moderate AS may either, mimic symptoms of sever AS resulting in misdiagnosis, or worsen the disease leading to higher mortality (Treibel et al., 2016). Therefore, these findings may warrant introduction of imaging techniques such as cardiac magnetic resonance imaging (MRI) and DPD scintigraphy in all patients with AS above the age of 75 routinely, to screen for presence of amyloid, as timely diagnosis of amyloid in such patients may change the course of intervention and reduce mortality.

1.5.3.3 Endomyocardial Fibro-elastosis

Endomyocardial fibro-elastosis (EFE) is a disease characterised by diffuse thickening of the endocardium resulting from proliferation of collagen and elastic fibers. Although, a cardiomyopathy on its own, it is often seen as a compensatory phenotype associated with other cardiac diseases. In the cases of presence of LV EFE in AVD, it is often regarded as the end stage form of LV remodeling, after initial dilatation and hypertrophy, although, other causes such as primary infection or obstructive lymphatics cannot be excluded. EFE is responsible for progressive loss of left ventricular diastolic function due to poor ventricular filling, and together with LVH, eventually leads to heart failure (Steger et al., 2012, Yarbrough et al., 2012).
Although, fibrosis and EFE can now be diagnosed by echocardiography and by means of cardiac MRI late gadolinium enhancement, endomyocardial biopsy still remains the gold standard for quantifying fibrosis (Oldershaw et al., 1980, Neubauer and Bull, 2017). Histopathological studies of the LV in AS showed presence of two types of fibrosis; interstitial fibrosis and replacement fibrosis. Interstitial fibrosis, due to an increase in collagen fibers within the cardiac interstitium, is often found in the reversible stages preceding the irreversible replacement fibrosis, occurring due to autophagy and oncosis, without any evidence of myocyte proliferation (Hein et al., 2003). This pattern of fibrosis is predominantly seen in the subendocardial layers as a result of decreased myocardial perfusion and increase in systolic wall stress (Anderson et al., 1979).

Studies show that presence of EFE leads to a deterioration in ejection fraction, along with an increase in myocardial stiffness. Although, the exact mechanisms responsible for this are unclear, it could possibly be due to the entrapment of muscle fibers in the fibrotic endocardium, or absence/reduction of endocardial related positive ionotropic effects on myocardial contractility (Villari et al., 1993).

Presence of EFE at time of surgery has been found to be an indicator of irreversible hypertrophy, and hence a marker of poorer prognosis. Patients with AS and severe fibrosis have a significantly worse 5-year survival than those with mild fibrosis (Azevedo et al., 2010). This further reiterates the need for a timely diagnosis of changes in the LV, with or without the presence of symptoms.

Although, the morphology of aortic valve disease has been studied in the past by a few groups, there is limited literature available providing the details of methodology and a comprehensible understanding of the results. Further studies are, therefore, needed to
be done to get closer to answering the wide array of questions arising from studying the morphology.

1.5.4 Morphology of AVD using imaging techniques

Imaging of the aortic valve is critical in establishing a diagnosis and severity of AVD, and the timing of valvular intervention. Furthermore, it assists in viewing the myocardial remodeling responses to these forms of disease (Dweck et al., 2012a). There are different non-invasive imaging techniques that help optimise assessments of AVD as well as the LV remodeling. These include the well-established echocardiography, and emerging role of computed tomography (CT) and cardiac MRI.

1.5.4.1 Echocardiography

Echocardiography (in particular transthoracic echocardiography (TTE)) is the key tool is the primary non-invasive imaging method for AS assessment, diagnosis, and evaluation (Baumgartner et al., 2010). Clinical decisions are made based on, accurate quantification of the disease severity assessed by imaging in context of presence (or absence) of symptoms. The course of AS progresses from aortic sclerosis to severe obstructive AS and is, therefore, viewed as a continuum. The recommendation for grading AS severity are seen in Table 1-4. Using this criterion, the values enable the clinician to assess the severity of AS based on imaging.

TTE is the initial diagnostic test carried out in patients with known or suspected valvular heart disease and can be viewed in both 2D and 3D. It allows for a comprehensive evaluation of valve morphology, including the number of leaflets (and raphe, if present), leaflet mobility, commissural fusion and valve calcification. Doppler echo on the other hand provides information on aortic valve dynamics, and the both are used conjointly to provide information on grading severity of AS. Whilst
the combination of TTE and Doppler echo is an effective approach in majority of the patients, there is a large potential for clinical confusion due to errors in measurements.

**Table 1-4: Recommendation for grading AS severity**

The AS severity grades ranging from aortic sclerosis to severe aortic stenosis based on the parameters adapted from ACC guidelines (Nishimura et al., 2014)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Aortic Sclerosis</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak velocity (m/s)</td>
<td>≤ 2.5</td>
<td>2.6-2.9</td>
<td>3.0-4.0</td>
<td>≥ 4.0</td>
</tr>
<tr>
<td>Mean gradient (mmHg)</td>
<td>-</td>
<td>&lt; 20</td>
<td>20-40</td>
<td>≥ 40</td>
</tr>
<tr>
<td>AVA (cm²)</td>
<td>-</td>
<td>&gt; 1.5</td>
<td>1.0-1.5</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Indexed AVA (cm²/m²)</td>
<td>-</td>
<td>&gt; 0.85</td>
<td>0.60-0.85</td>
<td>&lt; 0.6</td>
</tr>
<tr>
<td>Velocity ratio</td>
<td>&gt; 0.5</td>
<td>0.25-0.50</td>
<td>&lt; 0.25</td>
<td></td>
</tr>
</tbody>
</table>

Echocardiography derived LV ejection fraction is used in guidelines to reflect LV systolic function. LVEF is an important indicator for valve intervention, as a LVEF < 50% is associated with poor outcome in surgery. Although, morphology of the LV, in terms of hypertrophy and fibrosis, is better studied using cardiac MRI, 2D Echo can provide basic information that may be useful in assessing LV changes, preoperatively, in asymptomatic patients or patients unable to undergo cardiac MRI for any reason (Devereux et al., 1997, Pouleur et al., 2008, Celebi et al., 2010).
Transesophageal echocardiography (TEE) is superior to TTE when assessing planimetry of the valve orifice on 2D imaging. Furthermore, since the aortic root, isn’t an annular structure, 3D TEE allows for a more accurate measurement of the perimeter and area, which is crucial for device implantation. TEE also helps greatly in the monitoring of intra-procedural deployment of the aortic valve during surgical and trans-catheter (aortic valve replacement) AVR, as well as an assessment of post-procedural complications (Lindroos et al., 1993, Reant et al., 2006, Jabbour et al., 2011).

TTE and Transesophageal echocardiography (TEE), combined with 2D, 3D and Doppler evaluation provides clinicians with extensive information, on morphology and aortic valve hemodynamics enabling them to make a clinically sound decision (Pouleur et al., 2008, Otto, 2015).

**Fetal/Pediatric Echocardiography**

The leading reason for referral for tertiary fetal cardiac evaluation is the suspicion of a structural heart abnormality seen on obstetric ultrasound (Allan et al., 1994), amongst others such as maternal metabolic disease, or a family history of CHD. Fetal echocardiography is generally performed at 18-22 weeks of gestation, but increasingly at 12-14 weeks for case with family history or other fetal anomalies such as increased nuchal translucency. Use of color doppler helps with providing additional information on valve function and patency of ventricular inflow and outflow tracts and valve and chamber size can be assessed both qualitatively and quantitatively. Although, on an initial scan, the fetal cardiologist may be able to exclude major anomalies with studies showing >90% yield indications for cardiac anomaly, with 100% sensitivity and >95% specificity in diagnosing broad spectrum CHD, fetal echo may not be able to diagnose
minor degrees of aortic valve obstruction, and a later scan in the second or third trimesters may occasionally reveal the development of aortic stenosis (Allan, 1995).

Although new techniques such as Fetal Intelligent Navigation Echocardiography (FINE) are emerging to increase sensitivity and specificity of diagnosis of CHD, evaluation of specific data markers crucial to the quantification of diseases such as AS and their prognosis has, yet, not been possible (Yeo et al., 2018). Similar difficulties are seen when 3D Echo is carried out postnatally. Furthermore, cardiologists often struggle to find an optimal probe size for TEE. While, there are probes that are successfully used in neonates $\leq 4$ kg undergoing cardiac surgery or in older children, there are identifiable risk factors for TEE probe insertion failure, in the cohort of patients in between both age groups. A weight-based algorithm may help determine patients at risk for probe insertion failure, and subsequently development of a wider variety of probes (Lam et al., 2001, Wellen et al., 2013, Guler et al., 2015).

1.5.4.2 Computed Tomography and Magnetic Resonance Imaging

Advanced imaging techniques such as cardiac MRI (CMR) and Computed Tomography (CT), although not used routinely for diagnosis, are preferred by clinicians in order to obtain a more detailed morphological and planimetric assessment of the aortic valve and coronary arteries analysis, especially preoperatively before trans-catheter aortic valve replacement/implantation (TAVR/TAVI) (Bax and Delgado, 2017).

Studies have shown that, when compared to Echo, CMR shows comparable measurements, and in fact, provides excellent planimetric information, including assessment of aortic valve area. CMR, furthermore, had the best specificity and sensitivity, for detection of AS, amongst other non-invasive imaging techniques, and
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had the lowest intra-inter observer variability (Kupfahl et al., 2004). In addition, CMR allows for assessment of myocardial remodeling in AS, which, when carried out prior to intervention, can act as a good predictor of surgical outcome. This is done through quantification of the extracellular volume (ECV) of the myocardium. ECV acts as a useful CMR biomarker for fibrosis especially in conditions where there is formation of myocardial edema and does not account for changes in capillary density that is associated with adverse remodeling (Moon et al., 2013).

Although, CMR provides accurate morphologic and planimetric information, it is not always a feasible approach, for example, it is not suitable for patients with implanted pacemakers or for claustrophobic patients who cannot withstand being in a hollow ‘tube’. On the other hand, CT provides comparable information with a high spatial resolution and shorter imaging time, and is therefore, used regularly. CT, in combination with positron emission technology (PET) has provided evidence establishing the association between inflammatory and calcific processes and AS progression (Dweck et al., 2012b, Marincheva-Savcheva et al., 2011). Furthermore, CT enables the accurate assessment of planimetry of the true cross-sectional area of the left ventricular outflow tract (LVOT), which, a study showed in about a third of cases of severe AS, has led to reevaluation and reclassification as ‘moderate AS’ (Clavel et al., 2013, Kamperidis et al., 2015).

**Pre-intervention CT**

CT is now well regarded as an imaging modality for work up of patients prior to TAVR. It allows for a detailed assessment of the aortic root and coronary imaging, at the same time allows evaluation of the ilio-femoral morphology, thereby providing
crucial information determining patient eligibility, access strategy and selection of a desirable prosthesis (Blanke et al., 2010).

CT allows for a 3D assessment of the aortic root providing detailed information of aortic root geometry and dimensions (Leon et al., 2010, Smith et al., 2011). This, thus, enables accurate prosthesis sizing, as compared to information gathered from 2D Echo, therefore, reducing the incidence of a paravalvular leak (Unbehaun et al., 2012). Information on aortic root geometry can help in suitable procedural projection angles, and thus, enhance procedural success and efficiency (Masson et al., 2009, Gurvitch et al., 2010). Moreover, CT angiography of the ilio-femoral vasculature assesses the feasibility of intervention based on vessel tortuosity, calcifications and vessel diameter (Vahanian et al., 2008, Athappan et al., 2013). Finally, the success of the procedure, evaluation of prosthesis positioning and identification of asymptomatic complications can be assessed by CT imaging at various intervals post procedure (Blanke et al., 2010, Delgado et al., 2010, Willson et al., 2012).

1.5.4.3 Ex-Vivo Novel Imaging techniques

In recent years, novel imaging techniques such as micro-focus CT and synchrotron phase contrast CT have been developed enabling a detailed internal and external morphological assessment of tissue specimens, particularly in early gestational fetal autopsy (Lombardi et al., 2014). The 3D visualisation in small hearts with microscopic detail without the need for dissection and damage to tissues, was previously not possible with conventional advanced imaging techniques such as MRI and CT.
**Micro-focus CT**

Micro-focus CT (micro-CT) is an essential tool for phenotyping and for elucidating diseases. Similar to a medical CT scanner micro-CT is based on an X-ray beam that irradiates the object of interest. Photosensitive detectors record the unabsorbed photon signals and produce a data set that is then processed and interpreted using specialized software. It is based on two main construction design principles. In one instance, the specimen being examined is fixed with the X-ray source and detectors orbiting around it. In the second (used for *ex vivo* specimens), the specimen is mounted on a rotary platform. The latter allows for adjustment of the “radiating source-to-object” and “object-to-detector” distance, thereby increasing resolution (Clark-Greuel et al., 2007).

In order for the specimen to be scanned, it is immersed in a tissue contrast agent, which generally is Lugol solution (Iodine Potassium Iodide). This allows for a clear resulting image where one can differentiate between tissues and tissue substrates (Gignac et al., 2016). Studies on human specimens have shown that iodine enhanced micro-CT scan allows for creation of highly accurate 3D representations of a wide array of complex congenital heart diseases without the need for injection of contrast agents that may distort tissue. Diagnostic accuracy of micro-CT of CHD in mouse models and isolated human hearts extracted at autopsy is more than 90% with a >95% concordance seen in morphological features in human samples. Further micro-CT imaging of animal models and human specimens could prove to be useful in educating surgeons and interventionists alike, prior to intervening on live patients (Stephenson et al., 2012, Hutchinson et al., 2016, Hutchinson et al., 2017).
‘Synchrotron’ X-ray phase-contrast imaging

Although, conventional X-ray CT is quicker, easier to use and widely accessible, it suffers from poor soft tissue differentiation, especially in fetal and pediatric samples (Arthurs et al., 2017). Propagation-based synchrotron X-ray phase-contrast imaging (XPCI) combats this problem as the image contrast is based on the phase shift induced by the specimen instead of its attenuation (Bravin et al., 2013).

Like micro-CT, synchrotron XPCI allows high resolution 3D imaging of human heart specimens without the need for destruction of tissue (Zamir et al., 2016, Tsukube et al., 2017). This is crucial as there are a limited number of cardiac specimens with CHD, so an imaging modality that doesn’t lead to destruction of tissue should be used (Kaneko et al., 2017).

In propagation-based synchrotron XPCI, specimens are placed few meters away from the detector, at the stage’s center of rotation. A 20keV X-ray beam generated from a synchrotron light source is directed towards it and acquisitions performed by rotating the samples in a tube containing deionized water (Garcia-Canadilla et al., 2018). Although, still in its early stage of development, proof of principle studies conducted using synchrotron XPCI have shown that relatively large unstained samples can be visualized with the required contrast for the purpose of clinical and diagnostic use, thus, synchrotron XPCI has the potential to be translated into clinical practice (Zamir et al., 2016, Garcia-Canadilla et al., 2018).
1.6 Treatment of Aortic Valve Disease throughout the life course

1.6.1 Fetal Cardiac Interventions

Many congenital heart defects can be identified in utero by fetal echocardiography. Though many such malformations occurring during early stages of development do not change during the course of the gestational period, a small group of lesions, encompassing either left or right outflow tract obstructions experience significant changes (Vigneswaran et al., 2018). These conditions could benefit by intervention during fetal life.

Fetal Cardiac Intervention (FCI) is a relatively new and evolving field combining the expertise of maternal-fetal health experts, interventional cardiologists and fetal ultrasound specialists amongst others (Gellis and Tworetzky, 2017, McElhinney et al., 2010, Yuan, 2014). The main aim of FCI is to modify the progression of the disease in utero and to modify the outcomes of the patient to more favorable ones thereby reducing mortality and morbidity.

FCI can be considered for the following conditions: critical AS with evolving hypoplastic left heart (eHLH), pulmonary stenosis with intact ventricular septum and evolving hypoplastic right heart (eHRH), and HLH syndrome with intact atrial septum (Schidlow et al., 2014). HLH syndrome (HLHS) is usually identified by fetal ultrasound during mid gestation and exhibits marked under development of left sided structures in the left chamber and is usually associated with valvar stenosis or atresia. Patients with marked LV hypoplasia are not amenable to FCI and require surgical intervention after birth. Though post-natal staged surgical intervention is improving, it is associated with morbidity and complications including, ventricular and valvar dysfunction, arrhythmia, neurocognitive impairment and stroke amongst others.
(Goldberg et al., 2011). Therefore, a time-critical judgement needs to be made assessing the risks and feasibility of intervention either before or after birth (Norwood et al., 1983, McQuillen et al., 2010).

1.6.1.1 Severe AS with eHLHS

Prenatally, aortic valve stenosis can lead to severe left ventricular abnormalities, including HLHS. Studies evaluating left ventricular dysfunction in patients with critical AS have shown that in some cases, HLHS develops since the left-sided structures fail to grow at the same rate as the gestational age (Simpson and Sharland, 1997). This is usually manifested as an amalgam of primary left ventricular endocardial fibroelastosis, critical AS and HLHS (Tworetzky and Marshall, 2004).

Patients with severe AS and eHLHS are candidates for fetal aortic valvuloplasty (FAV). The aim of the intervention is to relieve aortic stenosis that causes hemodynamic alterations and achieve biventricular circulation after birth.

AS with eHLHS is a dynamic entity where a normally sized or dilated LV can evolve over a few weeks, resulting in severe HLHS (Simpson and Sharland, 1997). The mechanisms for this are unknown, however, this maybe as a result of a triggered genetic switch where myocytes differentiate into a non-proliferative state either as a direct result to increased LV afterload, or decreased myocyte perfusion, thereby, leading to cell death and cessation of LV growth (Schidlow et al., 2014) Intervention is only considered when there is a possibility of interrupting this sequence and benefitting the patient. Appropriate assessment and selection of patients for intervention is, therefore, necessary along-with proper counselling of parents regarding the benefits, and risks of FCI to the developing fetus which include possible fetal demise (Mizrahi-Arnaud et al., 2007, Schidlow et al., 2017).
Patient assessment and selection

The two primary considerations for intervention are, severe AS with features that are suggestive towards progression of HLHS, and the potential benefit of intervention, specifically biventricular circulation.

Echocardiography and Doppler helps to identify the features of severe AS with eHLHS. These include; (1) thickened and doming aortic valve, (2) small jet of antegrade aortic valve flow, (3) dilation of LV ventricle with multiple areas of echogenicity (representative of LV EFE), (4) diminished tissue velocities of the LV wall, and (5) Mitral regurgitation, amongst others. Retrograde transverse aortic arch flow, left-to-right flow across the foramen ovale, and significant LV systolic dysfunction are indicative of progression to HLHS. As to whether the left heart is salvageable, the criterion followed at well-known tertiary care centers are, normal sized mitral valve, a normal sized to dilated left ventricle, a left ventricle that is still capable of generating significant pressure and a LV that does not have a significant endocardial fibroelastosis.

Procedure, Outcomes and Risks

The procedure is carried out after appropriate fetal positioning and adequate maternal anesthesia, by insertion of an ultrasound guided cannula through the LV apex. Once the left ventricle is punctured, the guide wire and coronary angioplasty catheter are positioned for balloon dilatation across the aortic valve. After a successful procedure, the ensemble is withdrawn from the heart (Tworetzky and Marshall, 2004, Mizrahi-Arnaud et al., 2007, Wohlmuth et al., 2014).

Hemodynamic studies on patients who have undergone fetal aortic valvuloplasty (FAV) show improvement in comparison to pre-FAV values. This includes, antegrade
transverse aortic arch flow, normal left-to-right patent foramen flow, increase in LV ejection fraction and antegrade systolic arch flow. The lattermost being the most important predictor of biventricular circulation at neonatal discharge (Prosnitz et al., 2018). Although, FAV prevents formation of a hypoplastic LV, studies on patients have shown that two thirds of the patients developed a dilated and hypertrophic LV, postoperatively, that is related to LV loading conditions imposed by valvar disease (Friedman et al., 2015). Furthermore, neurodevelopmental delay has been noted in patients that have underwent FAV, however, this is similarly also seen in patients with HLHS without fetal intervention, suggesting that innate factors play a role for such outcomes (Laraja et al., 2017). Hopefully, with further advancements in surgical techniques and understanding of AVD both in fetal and post-natal life, there will be a larger proportion of patients maintaining biventricular circulation, and an even improved survival rate.

In general, percutaneous FCI is considered to be a safe procedure, with minor risk to fetal life and minimal maternal risk. However, fetal demise occurs in approximately 10-17 %. Several fetuses experience short duration complications such as, bradycardia and ventricular dysfunction (Freud and Tworetzky, 2016, Wohlmuth et al., 2014). So far, no maternal mortality has been reported following undergoing an FCI (Schidlow et al., 2017, Wohlmuth et al., 2014).

### 1.6.2 Surgical treatment of Aortic Valve Disease in infants and adults

If left untreated, heart valve disease, can result in backflow of blood which leads to further complications such as, arrhythmias, pulmonary hypertension cardiac hypertrophy and even cardiac failure (Cross and Work, 2015). Treatment of valvular heart disease involves valvar repair or replacement of the diseased valve. Often the
choice of repair versus replacement is a dilemma that surgeons and patients have to face, as they each have their own benefits and risks.

Aortic valve replacement (AVR) has presided as the most common form of valve surgery (Yacoub et al., 2014), where the diseased valve is cut and a prosthetic valve (made from either biological or non-biological source) positioned in its place. The placement of a prosthetic valve comes at a price, be it bio-prosthetic or mechanical. One, cannot deny the effectiveness of nature’s own material and design, and it is therefore, that efforts are being made to repair and preserve the original valve rather than replace it with man-made alternatives.

1.6.2.1 The Ross Procedure

One of the most famous surgical techniques developed in aortic valve repair has been the ‘Ross Procedure’ named after the cardiac surgeon, David Ross who invented it in 1964. The procedure gained popularity in the early 1990s and involves replacement of a diseased aortic valve with a pulmonary autograft, and placement of a homograft in the pulmonary portion (Stelzer, 2011).

The salient features of the Ross Procedure that make it different to the conventional AVR are as follows: (1) dissection of the aortic root (2) mobilisation of the coronary arteries (3) harvesting the pulmonary autograft (4) proximal autograft anastomosis (5) coronary artery re-implantation, and (6) pulmonary homograft implantation. The most widely used variant of the surgery involves root replacement rather than the sub coronary technique. This is due the different dimensions and commissural distributions between the aortic and pulmonary valve, making it more feasible to replace the root in its entirety (Conklin and Reardon, 2001, Mazine et al., 2018).
1.6.2.2 Treatment of AS in pediatric patients

Preservation of native aortic valve tissue has more rationale in pediatric patients, due to the growth potential of the native valve, the elimination of the need for anticoagulation therapy, and, the possibility for future valve replacement, if needed. Furthermore, the cause for aortic stenosis in pediatric patients are either bileaflet/unileaflet valve plus hypoplasia of the aortic root. Therefore, various techniques of aortic root enlargement are often incorporated to surgeries like The Ross Procedure. These different modifications include The Ross-Konno, Modified Ross-Konno etc. (Mavroudis et al., 2014, Mavroudis et al., 2009).

Currently, there are two methods by which obstruction can be relieved in patients with neonatal AS, balloon valvuloplasty and surgical valvotomy. Balloon aortic valvuloplasty is a minimally invasive procedure using a trans-femoral catheter. Recently, the procedure has been adapted using a smaller size (3-4 ‘French’) vascular sheath and can be safely performed on neonates (Stapleton, 2014). Both balloon valvuloplasty and surgical valvotomy are palliative treatments, the goal being to delay AVR until the patient is able to receive an adult sized cardiac valve with both procedures sharing similar success rates and no one having significant advantage over the other. Although, that being said, surgical valvotomy is still considered the preferred treatment of choice in cases where the stenosis is complicated with other conditions (Benson, 2016).

1.6.3 Prosthetic and Bio-prosthetic Heart Valves

Patients affected by valve disease require heart valve repair or replacement surgeries. Approximately 275,000 heart valves replacements are carried out worldwide annually (Zilla et al., 2008, Manji et al., 2012c). There are currently, 2 types of valves used for
heart valve replacement, mechanical heart valves (MHVs) e.g. SJM RegentTM heart valve (St Jude Medical Inc., USA) or bio-prosthetic heart valves (BHV) e.g. surgical bio-prosthetic valve (Edwards Lifesciences, CA, USA), and trans-catheter porcine valve (Medtronic, CA, USA) (Adams et al., 2014, Cohen et al., 2010).

1.6.3.1 Mechanical Heart Valves

MHVs, are made using a variety of synthetic materials such as pyrolytic carbon, Teflon (PTFE) and metal. When exposed to blood circulation, these synthetic surfaces activate complement and coagulation pathways leading to thrombosis (Nilsson et al., 2010). In order to prevent thromboembolism, patients with MHVs require life-long anticoagulation with vitamin K antagonists i.e. warfarin (Roudaut, Serri and Lafitte, 2007). Regular monitoring check-ups are done by measuring International Normalized Ratio (INR) to ensure an optimal dose is achieved, since higher doses carry the risk of spontaneous bleeding and a lower dose could induce thromboembolism (American College of Cardiology/American Heart Association Task Force on Practice et al., 2006). Thromboembolic complications remain an important cause of morbidity and mortality in patients with MHVs with an estimated incidence of 0.6 - 2.3 % per patient-per year (Vesey and Otto, 2004).

Despite the need for lifelong anticoagulation associated with MHVs, these valves are widely used due to their high durability. A long-term survival rate of 40 years has been reported with the use of an MHV, the Starr-Edwards valve (Grunkemeier et al., 2012). These valves are commonly used in, patients < 65 years of age and having a long-life expectancy, patients already receiving anticoagulation and/or where the patient is at risk of accelerated bio-prosthesis structural valve degeneration (SVD) due to factors such as young age or renal insufficiency (Pibarot and Dumesnil, 2009). MHVs are,
however, not ideal in younger patients that may be prone to injury, menstruating or pregnant females and in patients in the developing world where close and regular monitoring of anticoagulation may be difficult (Manji et al., 2012b).

### 1.6.3.2 Bio-prosthetic Heart Valves

BHVs are made using biological tissue derived from human cadaveric heart valves (homografts) or fashioned from chemically fixed non-vital bovine pericardium or porcine heart valves (heterografts or xenografts) (Simionescu, 2004). Due to the general shortage of human organ donors and an increase in need for valve replacement surgery, animal tissues have become the major source of materials for BHVs. There are two main types of BHVs, surgical valves requiring open-heart surgery and percutaneous transcatheter valves that do not require surgery. In most surgical valves, the leaflets of the valve are made from animal tissue and are mounted on a synthetic stent and surrounded by a sewing ring at their base (Schoen and Levy, 1999). Percutaneous valves, made either with self-expanding nitinol wire frame or cobalt-chromium balloon expandable frame with a sutured xenograft valve, have been developed; this structural modification allows the valve to completely fold on itself during implantation (Sinning et al., 2012). Percutaneous valves are preferred over surgical valves in cases of aortic valve replacement in a select number of patients that cannot safely undergo open heart surgery (Taramasso et al., 2014). In comparison to stentless valves, percutaneous valves also offer better hemodynamic performance and reduce mechanical stress due to increased orifice area (Finkelstein et al., 2014).

Initially, animal heart valves were fixed with formalin for sterilisation and preservation of the tissue (Ionescu, 1968). In early trials, formalin fixed heterografts exhibited a high rate of failure characterised by mononuclear and multinuclear cell infiltration.
The histology of explanted failed heterografts revealed stretching and deformation of the valvular cusps as a result of denaturation of collagen post implantation (Rose, 1972). The failure mechanism suggested a need for greater tissue preservation. Glutaraldehyde acts as fixing agent by forming cross links between amino acids in proteins through the reaction of its free aldehyde groups and primary amines. This strengthens the valve and also acts as a steriliser. Treatment of heterografts with glutaraldehyde allowed effective use of animal heart valves (Albert et al., 1976). Glutaraldehyde is the universal fixative currently used in commercial BHVs (Olde Damink et al., 1995).

The main advantage of BHVs compared to MHVs is that BHVs maintain a low rate of thromboembolism generally without anticoagulation. BHVs are durable in adults (>65 years of age) with a failure rate of only 10% within 10 years. BHV durability is significantly decreased in younger patients (<35 years of age), where a failure rate of nearly 100% in 5 years is seen due to premature structural valve degeneration (SVD) (Schoen and Levy, 1999). SVD is generally characterised by a high level of tissue calcification which promotes cusp tears and thickening and stenosis (Isihara, 1981, Schoen et al., 1987, Butany et al., 2007). Pannus formation, inflammation, and thrombus formation both grossly and microscopically also contribute to SVD (Butany et al., 2011). These pathologies can lead to valve stenosis through excessive growth of calcium nodules and regurgitation due to leaflet tearing.

If untreated, SVD can result in left ventricular hypertrophy and ischemia requiring patients with BHVs to undergo additional valve replacement surgery, or percutaneous valve-in-valve procedures. SVD caused valvular regurgitation is the most frequent cause of re-operative valve replacement in patients with BHVs (Walther et al., 2007).
1.6.3.3 Mechanisms of Structural Valve Degeneration of BHVs

Multiple factors contribute to tissue calcification in SVD including mechanical injury, fixation, lipid insudation, and immune injury (Pohle et al., 2001, Zilla et al., 2008, Ruzmetov et al., 2015).

Mechanical injury and SVD

Calcification is associated with valvular commissures and points of maximal flexion which receive high mechanical stress. This suggests that mechanical injury in these areas weakens the structural integrity and thereby promotes calcification which can result in leaflet tears. Sites of high mechanical stress are also associated with high concentration of inflammatory infiltrates found in these areas (Pohle et al., 2001, Ruzmetov et al., 2015, Zilla et al., 2008). Whether these inflammatory infiltrates contribute to SVD or are present in response to SVD is unknown.

Glutaraldehyde fixation

Glutaraldehyde fixation of BHVs is essential but may also promote calcification of BHVs by: forming cross links proteins due to the presence of unreacted cytotoxic aldehyde groups, and increasing plasma-lemma permeability to calcium by possibly inactivating the ATPase enzyme (Kim, 1999).

Immunological factors

Implantation of heterografts, such as porcine valves, can result in the development of SVD of the valve, characterised by calcification, pannus formation and valve tears. These valves also show signs of inflammation when explanted. This inflammation is characterised by stripping off of endothelial cells which exposes the sub-endothelial fibrous tissue, fibroblasts and platelet deposits along with macrophages which
contributed to destroying the collagen framework of the valves. More severe immune responses are seen in young adults and children as they have a stronger immune system than older patients (Manji et al., 2012b). Studies have shown mortality of up to 30% in pediatric patients that have been transplanted with BHVs and the actuarial survival rate after 5 years of operation is only 50% (Guvendik et al., 1989). In valves explanted that have undergone SVD, the pathology is often extremely advanced, and it becomes unclear whether the inflammatory cells that are present are the cause of the pathology or their presence is in response to the damaged tissue.

Studies on explanted BHVs in temporary circulatory assist devices such as, left ventricular assist devices (LVAD), which are used for a few months as a bridge to transplant, provide a view on the early histopathological changes, prior to clinical dysfunction. These valves have exhibited deposits of fibrin, macrophages, and neutrophil infiltration on the cusps and also demonstrate presence of IgG and complement proteins, platelet aggregation, micro-thrombi and surface deposition of complement factors and antibodies (Khan et al., 2008). These immuno-histological findings are consistent with an antibody mediated inflammatory process which can contribute to SVD, suggesting an early immune injury to the implant.

**Changes in design and processing for improved BHV durability**

In order to prevent BHV calcification and degradation and increase their durability, changes in stent design, chemical fixation and post fixation processing have been tested. Introduction of low-profile valves by Liotta-Bioimplant manufactured by Barone Laboratories, was aimed at facilitating implantation during mitral valve replacement (Liotta, 1982). In these low-profile valves, the supporting stent was sewn to the aortic annulus thereby decreasing the height to diameter ratio of the valve. This
redesign resulted in increased mechanical stress leading to enhanced calcification and leaflet tears (Singhal et al., 2013). Despite some instances of failures, there are still low-profile valves present in the market. One such valve is the Carpentier-Edwards Perimount magna mitral valve bioprosthesis (Edwards Inc., USA). This valve has shown to have reduced SVD, good short-term survival and excellent hemodynamics (Loor, 2016).

**Post-fixation processing changes in BHVs to reduce calcification**

Calcification of BHVs plays an important role in SVD and therefore measures need to be taken to reduce calcification. Animal models are used to test the effectiveness of new tissue fixation and processing methods and their effect on tissue antigenicity and calcification. The most common models involve subcutaneous implantation of fixed tissues in rabbits, rodents and juvenile sheep (Zilla et al., 2001, Leong et al., 2013).

A common anti-calcification treatment is based on the removal of the phospholipids which promote calcification. Post-glutaraldehyde fixation treatment with ethanol, formalin, and Tween-80 (FET) shows decreased calcification levels and is currently used as an anti-calcification treatment for Carpentier-Edwards valves (Girardot, 1994, Shen, 2001).

Although, these changes in structural design and processing protocols have been shown to reduce calcification levels in animal models and some have been adopted as standard practice in manufacture of clinical BHVs, they have not in general improved BHV durability significantly in younger patients and do not prevent age-dependent SVD. This suggests that there is another key element that contributes to the SVD of BHVs.
1.7 **Rejection of Bio-Prosthetic Valves**

In xenotransplantation when tissues are transplanted between members of different species such as, the implantation of a heterograft bioprosthetic valve in humans, a hyper-acute rejection (HAR) of the xenograft can occur within 24 hours post transplantation, due to widespread antibody-mediated complement activation (Rose and Cooper, 2000). If HAR is prevented, there is still the risk of delayed xenograft rejection (DXR) occurring over weeks and months due to secretion of platelet activation factors and type II endothelial cell activation (Hancock, 1997). Studies conducted in nonhuman primates (NHPs) looking at DXR have found presence of xenogeneic glycans in pig tissues that have pre-formed or induced antibodies in humans and NHPs which contribute to xenograft rejection.

Although, due to several processing reforms, HAR post-BHV replacement is unlikely, studies have shown a transient induction of anti-Gal IgM antibodies post-BHV replacement (Konakci et al., 2005, Manji et al., 2012a). In pediatric patients transplanted with BHVs, a more marked immune response, demonstrated by the induction of antibodies is seen, with the anti-Gal IgG antibody response more prolonged and pronounced after 2 months post-transplantation (Park et al., 2011). This antibody-mediated activation may be due to the presence of glycans on xenograft bio-prosthetic heart valves which trigger an immune response when transplanted in humans.

1.7.1 **Antibody Reactivity to Xenogeneic Glycans**

1.7.1.1 **Gal antigen**

Xenograft rejection was initially caused by abundant human antibodies that bind to the well-known xenogeneic glycan, galactose-α-1,3-galactose (Gal) (Sandrin and
McKenzie, 1994). Gal is synthesised by the enzyme, α-1,3-galactosyl transferase which is encoded by the \textit{GGTA-1} gene. As the \textit{GGTA-1} gene is inactive in humans, humans do not express Gal; however, high levels of anti-Gal antibodies (which exist in various forms of isotypes e.g. IgM, IgG) are present in humans as a result of an immunological response to antigenic stimulation by bacteria of the normal flora (Galili, 2013).

Studies have shown that binding of human anti-Gal antibodies, to Gal+ fixed bio-prosthetic tissue enhances calcification in rodent and rabbit subcutaneous implant models suggesting that circulating antibodies bound to fixed tissue contributes to bio-prosthetic graft calcification (Human and Zilla, 2001, McGregor et al., 2011).

Anti-Gal antibody induced rejection of porcine valves has now been prevented in genetically modified Gal-knockout (GTKO) pig models by inducing a mutation in the \textit{GGTA-1} gene so that the enzyme is no longer active, and therefore do not express the Gal antigen (Lai, 2002, Phelps et al., 2003).

Xenotransplantation of GTKO organs in NHP models did not induce an anti-Gal response and GTKO pigs lost tolerance to Gal antigen and now produce anti-Gal antibodies (Kiernan et al., 2008). Thus, there has been a complete elimination of the Gal antigen in GTKO pig tissues (Byrne and McGregor, 2012). Although, use of these GTKO donor organs eliminated anti-Gal mediated xenograft rejection, it did not fully prevent HAR and has not prevented DXR which now occurs due to pre-formed antibodies present against non-Gal glycans, Neu5Gc and Sda.
1.7.1.2 Neu5Gc

N-glycolylneuraminic acid (Neu5Gc) is a non-Gal xenogeneic glycan present on BHVs. It is synthesised from a sialic acid, N-acetylleuraminic acid (Neu5Ac) by the cytidine monophospho-N-acetylleuraminic acid hydroxylase (CMAH) gene. Neu5Gc is not produced in humans and is, therefore, recognized as a foreign antigen by circulating antibodies in humans.

Presence of Neu5Gc on commercially produced BHVs, and bovine and porcine pericardium was revealed through immunohistochemistry. An analysis of highly-specific affinity-purified human anti-Neu5Gc IgG, showed that anti-Neu5Gc strongly bound to the BHVs tested, signifying the immunogenicity of Neu5Gc on bio-prosthetic devices (Reuven et al., 2016). The McGregor group used immunofluorescent staining of pig pericardium to look for expression of all Gal, Neu5Gc and B4GALNT2. Pig pericardium was found to express all three glycans (Figure 15) (Byrne et al., 2015).

1.7.1.3 Sda antigen

One of the non-Gal xenogeneic glycan is the Sda blood group antigen which is a known minor blood group antigen, discovered in 1967 (Renton et al., 1967). In humans, the β-1,4-N-acetylgalactosaminyl transferase-2 (B4GALNT2) gene catalyses the last step in the synthesis of the Sda blood group antigen.

The role of Sda as an immunogenic xenoglycan was established through an analysis of induced antibody responses to non-Gal antigens in a pig-to-baboon cardiac xenotransplantation. Screening of the GTKO cDNA library obtained from human embryonic kidney cells (HEKs) using IgG induced after pig-to-baboon cardiac xenotransplantation identified the porcine B4GALNT2 gene responsible for antibody binding to these cells. This was confirmed by an increase in complement mediated
lysis produced by the B4GALNT2 expressing HEK cells and suggested B4GALNT2 produces a non-Gal glycan which is immunogenic in cardiac xenotransplantation and contributes to the observed immune response (Byrne et al., 2011, Byrne et al., 2014).

Figure 15: Immunofluorescent staining of pig pericardium to confirm presence of xenogeneic glycans.


Human antibodies reacting against Gal, Neu5Gc, and Sda constitute the majority of pre-formed human anti-pig antibody reactivity. Wang et al. studied different types of pig tissues in which the genes responsible for synthesis of these glycans, GGTA-1, CMAH and B4GALNT2 were knocked out to test for immunogenicity of these glycans. He compared levels of human IgG and IgM binding in RBCs isolated from Gal⁺, Gal-
KO, Double KO and Triple KO pig tissues. Both antibody isotypes are raised in Gal\(^+\) tissue, however a 4-fold decrease is seen in both IgG and IgM levels when GTKO tissue is used and a progressive decrease seen in these antibodies when double KO and triple KO tissues are used (Wang et al., 2017b). This suggests that the bulk of human anti-pig antibody reactivity is due to the presence of human antibodies present against all three glycans which might affect durability of BHVs.

1.7.2 Sda blood group antigen

Sda antigen, the main xenogeneic glycan looked in this thesis, is a high frequency poly-agglutinated minor blood group antigen produced by B4GALNT2. The structure of Sda is as follows:

GalNAc-\(\beta\)-1,4(Neu5Ac-\(\alpha\)-2,3) Gal-\(\beta\)-1,4-GlcNAc-\(\beta\)-1,3-Gal

In humans, Sda expression on RBCs ranges from: Sda (-) with no Sda antigen, present in 4% of the population; the more common Sda (+) with normal Sda levels in 90% of the population; to the rare Sda (++) individuals, with high Sda levels, called the Super Sda present in approximately 1% of the population (Dall'Olio et al., 2014). The genetic basis of variability in Sda expression is unknown.

In contrast to most RBCs from humans (Sd- or Sd+), RBCS from Sda (++) patients are poly-agglutinable by most human sera indicating that most people, with variable, moderate levels of Sda on RBCs (Sda +) produce antibodies against Sda. Anti-Sda is a complement fixing IgM cold agglutinin. Human serum contains variable levels of anti-Sda antibody (Spalter et al., 1999, Morton et al., 1970). Sera from Sda ++ individuals do not agglutinate CAD RBCs, and don’t make anti-Sda antibody suggesting possible immune-tolerance to Sda.
Despite the common presence of anti-Sda antibody and variable levels of Sda on RBCs, anti-Sda is clinically insignificant in terms of transfusion since it is most unlikely that it could cause a hemolytic transfer reaction. This is because, the level of Sda antigens on their blood cells is low and anti-Sda antibody in serum is poorly reactive at normal body temperature, as the anti-Sda antibody is a cold agglutinin.

1.7.3 B4GALNT2 polymorphism in different species

In humans, the molecular basis of Sda antigen polymorphism is not known, however in other species, the genetic basis for polymorphism is known or suspected e.g. mice and chimpanzees (Mohlke et al., 1999).

1.7.3.1 Polymorphism in mice

The majority of murine and bovine species show overall restriction of Sda expression to epithelial cells, which is similar to the location of expression in humans. Some strains of mice exhibit endothelial expression of B4GALNT2. These strains of mice, illustrated by DDK and RIIIIS/J mice, have lost gastrointestinal expression of Sda in favor of widespread vascular endothelial cell expression (Ponder and Wilkinson, 1983). The histologic polymorphism correlates with the mutation “Mvwfl” and a change in B4GALNT2 expression (Mohlke et al., 1999). Vascular endothelial cell expression of B4GALNT2 causes Sda modification of von-Willebrand factor (vWF) which is expressed by endothelial cells. This modified vWF is secreted inefficiently and is rapidly degraded in the plasma leading to a 20-fold reduction in vWF level in these mice when compared to other inbred strains (Sweeney et al., 1990). The molecular basis of this murine polymorphism is due to a regulatory polymorphism that correlates with loss of gastrointestinal epithelial expression of B4GALNT2 (Johnsen et al., 2008). As the Mvwfl1 polymorphisms has been observed in wild
derived mice the B4GALNT2 locus appears to be under natural active selection in these populations. Since B4GALNT2 knockout mice show a significant change in gut microbiota, which lowers their susceptibility to infection, it maybe that the Sda glycan play important roles in host-microbe interactions.

### 1.7.3.2 Polymorphism in pigs

Pigs express B4GALNT2 in endothelial cells (Wang et al., 2016). The expression of porcine B4GALNT2 in endothelial cells is different from humans, where B4GALNT2 and Sda are mainly found in gastrointestinal epithelial cells. Porcine expression of B4GALNT2 and Sda is similar to that seen in the *Mvwfl* mice. In order to learn more about the potential for polymorphic expression in pigs, different unrelated porcine inbred strains have been tested for B4GALNT2 expression. There are a wide variety of pigs used in agriculture and a number of regional variations, however, it is not clear if there is polymorphic expression of B4GALNT2 in pigs. A naturally occurring B4GALNT2 polymorphism in pigs would be useful if it prevented the localization of Sda in endothelial cells, as these animals could be used for the manufacture of a new generation of Sda free BHVs. DBA-based immunohistochemical staining has shown to be the most reliable marker of endothelial cells, since it strongly binds to vascular endothelial cells as shown in hairless Yucatan pigs, micro- pigs and also large breed agricultural pigs (Darr et al., 1990, Skowasch et al., 2006). No additional information of B4GALNT2 expression is known. Since DBA binds to Sda, these results suggest that this vascular expression of B4GALNT2 is common in pigs.

A strain of pigs, Gottingen Mini Pigs (GMP), have been specifically bred for use in biomedical research by a crossbreed of the Minnesota mini pig and the Vietnamese potbelly pig and further crossbred with the German landrace pig and are quite distinct
from commercial pigs in terms of smaller growth rate and smaller size. Since these pigs have not been a part of the commercial breeding genetics since 1960s, this strain would be a good candidate to have B4GALNT2 polymorphism.

With advantages and disadvantages of both valve repair and replacement, the question arises on whether valve repair procedures such as the ‘Ross Procedure’ or current prosthetic materials used for development of valves should be relied on completely. New approaches are needed to identify a durable biological source for production of bio-prosthetic valves, whilst at the same time, increasing our understanding of developmental processes that lead to the specific morphological changes associated with a malformed valve which would support advancements in surgical techniques for valvar repair.
1.8 **Hypotheses and aims**

1.8.1 **Hypotheses**

While the etiologies, pathogenesis, mechanisms of aortic valve stenosis differ throughout life course, there are morphologic and genetic factors that are common to all age groups. Understanding of these common factors is useful for the development of surgical techniques as well as design of durable bio-prosthetic valves and devices. Moreover, the assessment of morphologic features will vary depending on whether assessed by gross examination or using high definition imaging techniques. These differences carry significance when making surgical decisions in aortic valve intervention. Furthermore, the development of a bioprosthetic source of tissue that is resistant to degeneration will be a significant addition to the surgical realm of aortic valve treatment. A laboratory inbred strain of pigs may prove to be a useful source of bioprosthetic valve development as they may exhibit polymorphism and not express the xenogeneic Sda antigen, found in wild type tissue, which contributes to the degeneration of bioprosthetic heart valves.

1.8.2 **Aims**

I will address my hypothesis by:

1. Review of literature on cardiac morphology (specifically aortic valve), congenital heart disease, congenital and acquired aortic stenosis, fetal and adult cardiac interventions, and degeneration of bio-prosthetic heart valves (particularly the effect on xenogeneic glycans).
2. Use of Gene Ontology to annotate a selected cohort of genes involved in aortic valve development to provide a tri-dimensional framework incorporating biological, cellular and functional components, to understand the genetic basis of aortic valve disease.

3. Morphologic analysis of aortic valve stenosis specimens from fetal life to adulthood within the HTA Licensed Cardiac Archive at UCL Institute of Cardiovascular Science. This includes: complete sequential segmental analysis of hearts; documentation of associated lesions; detailed analysis of the aortic root and valve in terms of leaflet morphology, nodularity, calcification, commissural fusion, interleaflet triangles, sinus and ascending aorta morphology.

4. Develop optimal staining techniques for animal heart specimens that will enable these to be viewed under novel imaging techniques without damage to the tissue. Use that technique, to stain human specimen, and perform Micro CT analysis of human specimen of aortic root and aortic valve for a microscopic analysis of valvar morphology & calcification, muscular/fibrous support and adjacent valvar structures.

5. Examine tissues obtained from the laboratory inbred strain, Gottingen mini pig (GMP), for evidence of polymorphic expression of the non-Gal antigen, Sda using immuno-histological analysis to evaluate its potential use as a source of bio-prosthetic heart valves lacking Sda.
Chapter 2

Materials and Methods
2.1 Gene Ontology

The Gene Ontology project is an in-silico project within my thesis that was used to annotate a selected cohort of genes that were involved in valve development, particularly aortic valve development. Gene Ontology provides us with a tri-dimensional framework incorporating biological, functional and cellular components to help gain a better understanding of genes. This unit explains the various online resources that were used to create GO annotations in line with GO consortium guidelines (Balakrishnan et al., 2013, Huntley et al., 2014).

2.1.1 Bioinformatics Tools

A variety of bioinformatic tools were used during this project to identify the articles and protein identifiers to annotate the GO terms associated with the protein records and additional contextual information to include in the annotations created. The most frequently used tools are described below, and the URLs are listed in Table 2-1.

Table 2-1: URL addresses for bioinformatics tools used in this project

<table>
<thead>
<tr>
<th>Bioinformatics tool</th>
<th>URL address and resource use</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><em>Browser for biomedical articles</em></td>
</tr>
<tr>
<td>UniProtKB</td>
<td><a href="https://www.uniprot.org">https://www.uniprot.org</a></td>
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<td></td>
<td><em>Browser for UniProt protein IDs</em></td>
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<td>QuickGO</td>
<td><a href="https://www.ebi.ac.uk/QuickGO/">https://www.ebi.ac.uk/QuickGO/</a></td>
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<tr>
<td></td>
<td><em>Browser for GO terms</em></td>
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</table>
### 1. Tools and Databases

<table>
<thead>
<tr>
<th>Database</th>
<th>Description</th>
<th>URL</th>
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</thead>
<tbody>
<tr>
<td>HGNC</td>
<td><em>Human approved gene symbols</em></td>
<td><a href="https://www.genenames.org">https://www.genenames.org</a></td>
</tr>
<tr>
<td>Ensembl</td>
<td><em>Genome browser, to assess orthology</em></td>
<td><a href="https://www.ensembl.org/index.html">https://www.ensembl.org/index.html</a></td>
</tr>
<tr>
<td>Protein2GO</td>
<td><em>EMBL-EBI GO curation tool</em></td>
<td>Private access</td>
</tr>
<tr>
<td>EMBL-EBI: Uberon</td>
<td><em>Browser for tissue ontology (UBERON) terms</em></td>
<td><a href="https://www.ebi.ac.uk/ols/ontologies/uberon">https://www.ebi.ac.uk/ols/ontologies/uberon</a></td>
</tr>
<tr>
<td>EMBL-EBI: Cell</td>
<td><em>Browser for cell ontology (CL) terms</em></td>
<td><a href="https://www.ebi.ac.uk/ols/ontologies/cl">https://www.ebi.ac.uk/ols/ontologies/cl</a></td>
</tr>
<tr>
<td>Ontology</td>
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<tr>
<td>Cytoscape</td>
<td><em>Network analysis tool</em></td>
<td><a href="https://cytoscape.org">https://cytoscape.org</a> (software available for download)</td>
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</tbody>
</table>

### 2.1.1.1 PubMed

PubMed is a public, browser based, search engine operated by the United States National Library of Medicine that provides access to the MEDLINE (Medical Literature Analysis and Retrieval System Online) database of abstracts and a bibliography of published literature on biomedical information and life-sciences. As of 2019, it comprises of more than 28 million scientific citations including life science journals and online books.
PubMed was used to obtain experimental articles that provided information on genes related to semilunar valve development, especially the aortic valve. Papers that studied human proteins were preferred, however, due to lack of adequate literature describing the role of human genes in valve development, functional studies using other species were selected for annotation to fill in the existing gaps in information.

2.1.1.2 UniProt KnowledgeBase (UniProtKB)

UniProtKB is a public, browser based, Universal Protein resource which acts as a central source of protein data across all species provided by Swiss-Prot, TrEMBL and PIR-PSD databases (Figure 16). It is developed by a consortium of the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics, and the Protein Information Resource. Depending on the nature of the curations, they are added to either of the two sections of the UniProtKB database, Swiss-Prot or TrEMBL.

Swiss-Prot contains manually reviewed, non-redundant protein annotations based on information obtained from scientific literature and computational analysis provided by bio-curators’ evaluation. TrEMBL contains un-reviewed automated protein annotations based on high-quality computational analysis of the literature and coding sequences provided by other databases such as EMBL-Bank, GenBank.

UniProtKB was used to identify the correct unique species-specific protein accession ID. To ensure maximum accuracy of protein accessions, information provided by Swiss-Prot was used, however, occasionally a Swiss-Prot manually curated record was not available, in these cases, protein accessions by TrEMBL were used.
UniProtKB is a public browser that provides comprehensive protein data. In the search box the name of the protein of interest is entered which will then yield all protein accession results. The ones with the Gold Star are reviewed and manually annotated Swiss-Prot proteins and have exclusively been used in this project. The unreviewed accessions are computationally analyzed listings by TrEMBL. The Entry field is the UniProtKB accession ID, which is used on other ontology portals (UniProtKB portal).

2.1.1.3 QuickGO

QuickGO is a public, browser-based tool that provides curators with information about a ‘GO term’ and protein GO annotations. Each GO term record in QuickGO includes the GO term unique GO:ID (a numerical identifier), along with its human-readable name and definition, and where it is places within one of three ontology aspects, cellular component, a molecular function or a biological process. Further information provided on Quick GO includes, ancestor chart, child terms, protein annotations and co-occurring GO terms. QuickGO could be searched by GO term, GO:ID, UniProtKB and/or a PMID (Figure 17).

QuickGO was used to select the most specific and accurate term for a protein annotation.
Figure 17: QuickGO interface.

QuickGO is a database providing users with information on all the existing annotations. Annotations are listed with a UniProtKB protein identification code, a human-readable symbol, evidence code and evidence source i.e. reference and the assigning body. A search on GO terms of interest will display all annotations made to that term. These results could be filtered based on Taxon, Gene products, Evidence Code etcetera. Furthermore, annotations made to child terms can be filtered as well depending on the user’s preference. This figure shows 70 annotations made to the GO term ‘aortic valve morphogenesis’ (QuickGO browser interface).

2.1.1.4 HGNC/HCOP

HGNC (HUGO Gene Nomenclature Committee) is a public, browser-based database, which serves as the worldwide authority that assigns standardised nomenclature to human genes. HGNC is responsible for approving a unique gene name and gene symbol, thus, allowing for clear, unambiguous references to genes within scientific communication and also enabling for electronic data retrieval from existing literature and databases.

HGNC was used as a resource for obtaining genetic information about genes related to valve development.
HCOP (HGNC Comparison of Orthology Predictions) is a tool provided by the HGNC, which uses 13 protein ortholog prediction tools to provide a list of predicted orthologies and detailed supporting evidence.

HCOP was used to verify strong 1-to-1 orthology between non-human proteins, (mouse, rat and pig proteins) annotated directly and their human orthologues which were annotated by curator-inferred sequence similarity.

2.1.1.5 Ensembl

Ensembl is a public, browser based, genome database which provides datasets and analysis tools that enable genomics. Ensembl provides a summary of the gene, including a unique Ensembl identifier, synonyms, location and corresponding UniProtKB ID. It also provides data on comparative genomics that calculates percentage of sequence similarity between known sequences and exact sequence identities.

Ensembl was used to obtain further information about a gene and find appropriate Ensembl Identifiers to include in annotation extensions. The compara tool was used to compare amino acid sequence arrangements and similarities between non-human (mouse, rat, pig) and human orthologues.

2.1.1.6 Protein2GO

Protein2GO is, a private, browser-based tool for authorised curators, used to submit annotations to the Gene Ontology Annotation database (Figure 18).

Information submitted on Protein2GO included,
**PubMed ID** – to identify the experimental paper where the annotations were obtained from.

**Entity ID** – UniProtKB accession, to identify the protein to which the annotation was made.

**GO ID** – to identify the GO term being annotated.

**Evidence** – which provided an ‘Evidence Code’ depending on the method of how the annotation was obtained.

There are some additional fields on the Protein2GO user interface;

**Qualifier** – which is used if an experiment demonstrates that a protein is ‘NOT’ involved in a process.

**With** – to indicate physical interaction between annotated entities.

**Extension** – this field was used to provide further details of the annotation.

**Transfer Annotation(s)** – was used to migrate annotations from non-human to human orthologues based on curator-inferred sequence similarity.

![Protein2GO interface](image)

**Figure 18: Protein2GO interface.**
Protein2GO is a private interface used by curators to submit annotations to the Gene Ontology Database. All the fields shown in pink are compulsory to complete an annotation. The UniProtKB protein identification code of each protein is entered into the field marked ‘Entry ID’, the GO term ID number is entered into its namesake field, the evidence code in the evidence field, and the evidence source i.e. Journal Article in the field labelled ‘Reference’. Optional fields include ‘Qualifier’, ‘With/From’ and ‘Extension’. There are three kinds of annotations that can be entered based on their type: process, function, component (Protein2GO Browser Interface).

2.1.1.7 EMBL-EBI: Uberon

Uberon is a public ontological database of anatomic structures in a variety of species including comprehensive relationships to taxon-specific anatomic ontologies.

The Ontology Lookup Service was used to search for appropriate Uberon terms to include in the annotation extension field and specify that the annotation created eas a cellular component, process or function that was detected in a specific anatomical structure.

2.1.1.8 EMBL-EBI: Cell Ontology

Cell Ontology is a public ontological database of cell types in different species. These terms (identified using the Ontology Lookup Service) were used to specify annotations that occurred in a particular cell line.

2.1.1.9 VLAD

VLAD (Visual Annotation Display) is a web user interface that allows users to input a customized set of Gene Ontology annotations and transforms it into a graphical and tabular representation of the Gene Ontology terms associated with the submitted query dataset.

VLAD was used to analyze the annotations that had been submitted for the prioritized list of proteins (Query Set) and to calculate the term enrichment associated with the Query Set.
2.1.1.10 Cytoscape

Cytoscape is a visualisation and analysis tool for genetic interactions and biological pathways. It is able to link these interactions with GO annotations to provide a visual representation of term enrichment by means of other application add-ons.

All 34 human proteins annotated as part of this project were used to create a network of proteins constructed using the Cytoscape v3.7.1. software, which allowed for a pictorial representation of these proteins. The proteins were analysed using a merger of different databases including, IntAct, BHF-UCL and UniProt, amongst others. Due to a wide range of different databases and repeated interactions between proteins, the network graph was modified by removing duplicate edges and self-loops which resulted in a neater looking network, and with the help of the ‘Network Analyzer’, the size of the ‘nodes’ (each representing a protein) in the network were mapped based on the number of interactions they shared with other proteins. The GO terms associated with this network were then identified using the in-app tool, GOlorize alongside the BinGO plugin. The following parameters were applied: Hypergeometric test, Benjamini & Hochberg False Discovery Rate correction, significance level 0.05, reference set (human proteome; obtained from the GO term ontology file available online (go-basic.obo)), and a list of published annotations within the GO database prior to starting the project and after completion of the project (gene_association.goa_human.177.gaf; 26/02/18 and gene_association.goa_human.gaf; 29/03/19, respectively). This analysis enlisted the list of GO-terms annotated to the list of proteins, starting with the most over-represented terms sorted with the smallest p-values (most significant) on top. In order to assess the degree of enrichment achieved by this project in relation to valve development, specifically aortic valve development, I selected 3 GO terms: Heart
Valve Development, Aortic Valve Development, and Endocardial Cushion Development. Each of these terms was colored differently, which allowed for the visualisation of nodes that have been significantly enriched under the listed terms.

2.1.2 Curation Process

2.1.2.1 Selection of Experimental Papers

A primary literature search was conducted by project supervisors, Prof Lovering and Prof Cook and by BHF biocurator, Dr Campbell to source experimental papers on genes involved in heart development. PubMed IDs for the selected papers were checked in Protein2GO to determine whether the paper was previously curated (Figure 19). Papers were prioritized for curation depending on the species and their relation to aortic valve development. Human genes playing a role in aortic valve disease were prioritised over non-human genes.

2.1.2.2 Manual Curation

Once a paper was selected for curation, the species-specific UniProtKB protein accession was obtained. The abstract was studied to provide an overview of the paper and highlight the focus of the experiments conducted. The text and figures were reviewed, and figures that presented a novel experimental evidence were curated, based on matching GO terms that were identified through QuickGO. The PubMed ID, UniProtKB protein accession, and the relevant GO terms were then added to the particular protein record in Protein2GO. Initially, all annotations submitted onto Protein2GO were submitted as a PRIV source (i.e. private - not viewed publicly on the GO database) and were reviewed by curators, Dr Huntley and Dr Campbell. Once approved, they were made public under the BHF-UCL source (British Heart Foundation, University College London group) (Figure 19).
It was crucial to select the most appropriate and specific GO term for each curation. This was ensured by reviewing ancestral charts and child terms of the GO terms before selecting the GO term.

2.1.2.3 Annotation Extensions

Annotation Extensions were added for all annotations, to provide a more detailed curation of the protein. Depending upon the extent of experimental evidence found, this involved, identifying the anatomical structure using ‘Uberon’, identifying cell type if appropriate using ‘Cell Ontology’, specifying the gene using ‘Ensembl’ whose expression was modified by the action of the protein being annotated and inclusion of any relevant biological process or cellular component GO terms that were relevant to the annotation.

2.1.2.4 Evidence Codes

Evidence codes are used to identify how the annotation to the particular GO term is supported. There were 4 evidence codes used in this project:

**IDA** – Inferred from Direct Assay, was used to indicate that a biological, or biochemical experiment was carried out to support a protein’s molecular function, role in biological process, or its cellular location.

**IMP** – Inferred from Mutant Phenotype, was used to indicate that function, process or cellular component inferred based on differences in function, process and cellular components observed in phenotypes where there was either a mutation in a single gene (in human species), knockouts, knockdowns and overexpression assays (in non-human species).
IGI – Inferred from Genetic Interaction, was used for annotations based on experiments reporting the effects of mutations to more than one gene. The interacting protein is recorded in the ‘With’ field when the IGI code is used.

IC – Inferred by Curator, was used in cases where an annotation was not supported by any direct experimental evidence but was inferred by me and crosschecked by senior biocurators, based on other GO annotations for which evidence was available.

ISS – Inferred from sequence or structural similarity, was used for all annotations that were curated to non-human species. This evidence code is based on the 1-to-1 orthology between species verified on HCOP. Curator inferred sequence similarity was demonstrated by HCOP and by comparison of orthologues in Ensembl to determine the presence of strong orthology between the non-human species (mouse, rat, pig) to human genes. The ISS code was only applied when there was greater than 80% amino acid sequence identity found between orthologues and it was verified with Ensembl that there was a 1-to1 orthology between all genes considered for an ISS annotation (Figure 20). Due to lack of sufficient literature on human genes involved in heart development, this evidence code was used to understand the genetic nature of many of the human genes, using experimental evidence found on non-human genes.
Figure 19: Flowchart highlighting the steps in manual curation of a paper

1. **Literature search from PubMed**
   (Read Abstract to see relevance with the project)

2. **Check PMID of paper in QuickGO**
   (Ensure paper has not been annotated)

3. **Read abstract and analyze figures**
   (Check which figures provide experimental evidence)

4. **Appraise ‘Methods’ Section**
   (Identify species for UniProtKB accession)

5. **Appraise ‘Results’ Section**
   (Identify appropriate GO terms on QuickGO)

6. **Assess appropriate evidence codes and appraise ‘Discussion’ for any co-occurring GO terms and information to add in annotation extension.**

7. **Upload annotations on Protein2GO as a PRIV file.**

   Once approved by curators, annotations are public under BHF-UCL source.
Figure 20: Algorithm for curating non-human species.

This algorithm can be used to ensure comprehensive annotations of human proteins. Non-human experimental data are often the most informative information available. Consequently, when non-human data is curated, the annotations are transferred to the orthologous human protein records to provide comprehensive annotation of the biological role of these proteins. The flowchart indicates the steps taken in this process.
2.2 **Methods used to conduct morphological studies**

2.2.1 **Gross Examination**

The Cardiac Archive at the Institute of Cardiovascular Science and Child Health, London, UK consists of 2,623 heart specimens (July 2019) obtained from the 1940s to the present day, from all age groups across the life-course; fetal life to adulthood. They exhibit a wide range of congenital and acquired cardiac abnormalities and are held under UK Human Tissue Authority Research License 12220. The specimens held in the archive have been formalin fixed and are kept in 3% formaldehyde solution in a temperature-controlled environment. Heart specimens eligible for inclusion in this thesis were those that had been diagnosed as having aortic stenosis, anatomically. Fetal, pediatric and adult specimens designated as having this condition were selected from the archive database after applying specific inclusion criteria, namely that the hearts that had not undergone aortic valve repair or replacement, still showed adequate preservation and had not undergone extensive previous dissection or histologic sampling. Detailed examination of these specimens followed the protocol described below. Finally, the age and sex of the specimens were retrieved where possible from available cardiac archive records and autopsy reports.

2.2.1.1 **Aortic Valve Examination**

Following gross inspection to check for presence of any visible deformity and surgical intervention, a thorough examination of the aortic valve was performed. The number of valve leaflets present in each specimen was determined. This was based on the number and size of leaflets, the fusion of commissures and number of sinuses. The valves were characterized accordingly: (1) uni-leaflet valves, with fusion of two commissures; (2) bi-leaflet valves, with fusion of one commissure and two leaflets of
unequal size and (3) tri-leaflet valves; with no fusion between commissures and 3 leaflets. In cases where there were 2 or 1 leaflets, the sites of fusion between leaflets were assessed and noted. In cases of fusion, the presence, number and composition of ‘raphe’ was recorded. The composition of the raphe was based on the presence of; fibrous aortic wall tissue which was in continuation with the wall and pale in color, characterized as ‘fibrous’, soft valvar tissue and pink in color, characterised as ‘valvar’, or in cases where both types of tissue was present, it was characterised as ‘fibrovalvar’. Using a Vernier caliper, the maximal thickness of the valve leaflet(s) was measured. The valve leaflets were then observed for presence of nodularity and calcification on arterial and ventricular surfaces. If calcification was present, they were assessed for degree of calcification from mild – moderate – severe depending on the surface area of the valve that they covered. Calcification was characterised as mild, moderate and severe depending on the area of the valve involved: less than 1/3\text{rd} of the valve, 1/3\text{rd} to 2/3\text{rd}, or more than 2/3\text{rd} of the valve, respectively. Any visible nodularity or calcification were noted.

Next, the area of each of the interleaflet triangles was calculated. For the purposes of this examination, an interleaflet triangle was defined as the fibrous component of the aortic root, between adjacent leaflets, that is bound by the semi-lunar attachments of the leaflet at each side, and by a base, formed by between the basal attachments of each sinus (Figure 21). A caliper was used to measure the base of each interleaflet triangle, and the height was determined by measuring the distance from the apex of the triangle to the midpoint and perpendicular to the base. In cases, where it was not possible to measure the dimensions, a loss of interleaflet triangle was noted. In order to measure the circumference of the ventriculo-arterial junction, the aorta was manipulated into a cylindrical shape and then using the upper ‘jaws’ of the Vernier
caliper the diameter was calculated, which was subsequently multiplied by $3.14 (\pi)$ to provide us with the circumference of the aortic root. A ratio of the area of interleaflet triangles to the cross-sectional area of the aorta was calculated and categorized according to the type of raphe. These values were then plotted using a box and whisker plot.

![Diagram of interleaflet triangles and aortic root](image)

**Figure 21: Dimensions of interleaflet triangles**

The dimensions of the interleaflet triangles used in some of the previous literature and those used in this thesis. The red triangle indicates the dimensions of the ILT formed by the sides of the leaflets and the anatomic ventriculoaortic junction as a base. This method has been used by some morphologists in the past. The blue triangle indicates the dimensions of the ILT measured in this thesis. Note the base of the ILT is the line between the basal points of attachment of two leaflets (Adapted from Fuster V, Hurst’s The Heart, 13th edition).

**2.2.1.2 Left Ventricular Examination**

Assessment of the left ventricular wall included measurement of both its length and thickness. Two independent techniques were assessed in order to measure the length of the left ventricular wall (Figure 22). In the first technique, I used a thread to measure the epicardial surface of the left ventricle, starting from the left atrioventricular junction farthest away from the septum (lateral wall) to the apex of the left ventricle. This technique was later disregarded as there was a greater chance of error since the specimens had been dissected in slightly differing planes and therefore determining
the same point of apex was difficult. The second technique was based on the parameters used in echocardiography (Gardin et al., 1995, Ghanem et al., 2006, Muraru et al., 2013), where the left ventricular length is measured between the mid-point of the mitral valve to the apex in the long axis of the ventricle. A thread was placed in a straight line from a mid-point between the two mitral valve leaflets to the apex. The measurements were replicated at two different time points using same observer and different observers and reproducibility was assessed using Bland-Altman plots for Intra- and Inter-observer variability. The thickness of the LV wall was measured using a Vernier caliper at a point 1/3rd of the total length of the LV from the apex of the heart (Figure 22). A thickness to length index was then calculated using thickness as a fraction of the length. The left ventricle was inspected grossly for evidence of endocardial fibro-elastosis (EFE). EFE was then characterized qualitatively as being either mild, moderate or extensive. Mild EFE involved pinkish-grey mild opacity of the endocardium which was often patchy in nature. Moderate EFE was defined when up-to half of the LV endocardium was opaque along with smoothening of the LV trabeculations. Extensive EFE was defined as opacity of more than half of the LV endocardium, smoothening of the LV trabeculations, and a firm or hard textured endocardium.
Figure 22: A visual representation of the left ventricular measurements taken in this thesis.

The green line is the length of the epicardial surface of the left ventricle from the left atrioventricular junction (lateral wall) to the apex of the left ventricle. This measurement was disregarded due to differences in anatomic dissection of the samples. The yellow line represents the technique opted to measure the length of the left ventricle. This was done from the midpoint of the mitral valve to the apex. The maximal thickness of the LV measured at 1/3rd of the length of the LV wall from the apex of the LV is represented by the purple line.

2.2.1.3 Statistical Analyses

Distribution of data was assessed using box and whisker plots that provided the mean, standard deviation, median, and the first and third quartile (q1, q3). In order to characterise results, and for proportions, Confidence Intervals (CI) were calculated using an online calculator (https://www.mathsisfun.com/data/confidence-interval-calculator.html) based on the formula:

\[
\text{Mean} \pm \text{Z-value} \times \left( \frac{\text{Standard Deviation}}{\sqrt{\text{number of samples}}} \right)
\]

The CI is the percentage of samples likely to exhibit a similar result in any given population. In order to calculate a 95% CI, a Z-value of 1.96 was used.
In order to calculate the significance of results, One-way ANOVA analysis was performed which yielded an $f$-ratio value and a $p$-value.

### 2.2.2 High Resolution Imaging studies

2 rabbit hearts, 2 lamb hearts and 4 human heart specimens (1 normal 24-week fetal heart, 2 abnormal 24-week fetal hearts, 1 abnormal adult heart) obtained from the ICS Cardiac archive were used to carry out imaging studies.

#### 2.2.2.1 Analysis using Micro-CT Imaging

**Absorption-based micro-CT imaging**

Scanning was carried out after 3, 21 and 28 days of immersion in iodine. Prior to each scan, the specimens were removed from the iodine solution, rinsed with distilled water to remove excess surface iodine and dried using a clean gauze. Specimens were secured in a low-density plastic cylinder with supporting material (gauze) to ensure mechanical stability during the X-ray examination. Absorption-based micro-CT image sequences were acquired with either a Med-X or X TH 225 ST micro-CT scanner with multi-metal target (Nikon Metrology, Tring, UK). X-ray energies, target material (tungsten or molybdenum) and current were optimized for each individual specimen to maximize detector saturation without filtering (energy, 100 kilovolts; current range, 90-100 microamps; power range, 9-10Watts; gain, 24dB). Exposure time was 354ms, with a range of 2-4 frames per projection. Scans were reconstructed using modified Feldkamp filtered back projection algorithms with proprietary software (CTPro3D; Nikon Metrology) and post-processed using VG Studio MAX (Volume Graphics GmbH, Heidelberg, Germany). Resulting geometric magnification size varied according to specimen size. Post-processing techniques involved gray-value windowing, a clipping technique designed to digitally remove supporting material, and
multiplanar reconstructions, to create virtual dissections of fetal hearts. Scan times were between 1-hour 5mins to 1-hour 14 mins.

Following micro-CT scanning after 28 days, the specimens were immersed in a 2% sodium thiosulphate solution overnight for iodine removal and then returned to a 3% formaldehyde solution.

2.2.2.2 Optimization of Techniques for Micro-focus CT imaging

Before imaging human heart specimens, two techniques for optimizing micro-CT imaging were assessed.

Fixation

Two fresh lamb specimens weighing approximately 250 g each were obtained from the local butchers, and washed thoroughly to remove any blood and clots, and later immersed in 10% formaldehyde to fix them. A minimum of 72 hours was allowed for fixation of hearts before further experiments were conducted.

Siliconization

Two experiments were conducted using a pair of lambs. 1 formaldehyde-fixed lamb heart specimen was siliconized using Silicone Rubber Compound, RS Pro Transparent Silicone Sealant Paste 100g tube (RS Stock No: 555-588). The silicon compound was injected into the heart, through the aortic and pulmonary trunks, using a 500ml syringe. The second heart was not siliconized to serve as a control. These hearts were then left at room temperature for 30 mins to allow the silicone to cure (as recommended by the manufacturer).
**Iodination**

To iodinate samples, each sample was subsequently immersed into 1 liter of 2.5% Iodine Potassium Iodide (I2KI) and formaldehyde. For each experiment, 1L of 5% I2KI solution was made by adding 100g of Potassium Iodide (Sigma-Aldrich SKU 793582) into a conical flask and dissolved in 100ml of water using a magnetic stirrer. The top of the flask was placed to ensure no solution escapes. 50g of Iodine (Sigma-Aldrich SKU 207772-M) was then added to the flask and made up to 1L (by adding 900 ml) with water. The solution was, again, stirred for 10 minutes using a magnetic stirrer. In order to make a 2.5% solution, 1L of 10% formaldehyde was added to 1L of 5% I2KI.

**2.2.2.3 Image Analysis and Segmentation**

Virtual cardiac anatomy analysis and aortic valve segmentations were performed on an Apple Mac Pro (Late 2013) with 3 GHz 8-core Intel Xeon E5 processor, 64 GB 1866 MHz DDR3 ECC RAM and AMD FirePro D300 2048MB graphics card. Micro-CT and SR-PCI data sets were first visualized using the image processing software Fiji. Visualization of the interleaflet triangles (ILTs), and the calculation of the area of ILTs was carried out on Dell PC with 2 2.3 GHz Intel Xeon processors, 256 GB RAM using the VGStudioMax software (Volume Graphics, Heidelberg, Germany).
2.3 Methods used to examine Sda antigen expression

Porcine strains

Tissues from pigs engineered with a mutation which knocks out the GGTA-1 gene are housed at the Royal Veterinary College (RVC) Bolton Park animal facilities. These GGTA-1 knockout pigs (GTKO) are covered under PPL 70/7123 for standard breeding and as a source of tissues for research. The project license holder is Melanie Jane Wood, Senior Study Director at the RVC. The animals were produced from somatic cells via method of somatic cell nuclear transfer which allows inactivation of specific genes, in this case the GGTA-1 gene.

Frozen and formalin fixed tissues from Gottingen mini-pigs (GMP) were kindly provided by Dr Barbara Kessler, Chair for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians University of Munich.

Tissues from these unrelated strains of pigs were used to test for the expression of B4GALNT2 gene.

2.3.1 Immunohistochemical staining

2.3.1.1 Tissue fixation and processing

Tissues from GTKO and GMP were fixed in formalin and embedded in paraffin for tissue sectioning and immunohistology. Fresh GTKO and GMP tissue was fixed for 48 hrs in 10% formalin at room temperature and then washed in PBS. Tissue embedding and sectioning was performed by IQ Path at the UCL Institute of Neurology.

Seven tissue samples, heart, lung, liver, kidney, spleen, small intestine, and large intestine from 3 different pigs were used. Two of them were GTKO pigs and 1 was a
GMP. The tissue sections were de-paraffinised by placing them for 3 minutes in histoclearing agent (Sigma-Aldrich Laboratories, H2779-1L) thrice followed by an ethanol series (100%, 95%, 80%, and 70%) with a distilled water rinse to rehydrate the tissue. Endogenous peroxidase activity in the tissue was blocked by incubating the sections with 10% Hydrogen Peroxide (Sigma-Aldrich Laboratories, 31642) for 60 seconds after which they were thoroughly washed with water. Antigen retrieval was performed by treating the sections for 20 minutes by placing them in a container which had Tris-EDTA buffer (pH 9.0) [10 mm Tris and 1mm EDTA] at 60°C and then removed and then run under cold tap water for 10 minutes.

2.3.1.2 Immunostaining

The samples were stained to evaluate presence of Sda antigen by analysing binding to the Dolichus Bilflourus Agglutinin (DBA) lectin which recognizes terminal epitopes of Sda antigen (Byrne et al., 2018). This was performed by incubating the slides in PBS for 10 minutes and then blocking with 1% bovine serum albumin (BSA) in DPBS (2.67 mM Potassium chloride, 1.67 mM Potassium phosphate monobasic, 137.93 mM Sodium chloride and 8.06 mM Sodium phosphate dibasic) for 30 minutes at room temperature. Sections were stained with biotin conjugated DBA (Vector Laboratories, B-1205) at 5μg/mL in 1% BSA in DPBS at 4°C overnight. Negative control slides remained in DPBS with 1% BSA in DPBS also at 4°C overnight.

After staining, the slides were washed 3 times with PBS for 3 minutes each and were then blocked with 1% BSA in DPBS for 30 minutes at room temperature. The slides were incubated with streptavidin conjugated to polymerized horse radish peroxidase (streptavidin poly-HRP) (1:500) (Thermo Scientific, PD200740) for 90 minutes at room temperature, washed 3 times with PBS for 2 minutes each time, incubated with
DAB (SIGMAFASTTM 3,3’- Diaminobenzidine tablets, D4293) for 15 minutes at room temperature. DAB produces a dark brown stain when DBA is bound which is evidence of presence of Sda antigen. The staining reaction was stopped by rinsing the slides well with water.

2.3.1.3 Histology staining

The DAB stained slides were counterstained for 3 minutes with Hematoxylin (Sigma-Aldrich Laboratories, GHS116), and rinsed with deionized water for 5 minutes. The slides were then dipped 10 times in acid ethanol to de-stain, followed by washing them with tap water then rinsing with deionised water twice for 1 minute each. The slides were dehydrated by passing through an ethanol series (70%, 80%, 95%, 100% and 100%) for 1 minute each. Excess ethanol was blotted before going into Histo Clearing agent after which the slides were removed, carefully wiped and mounted using a drop of mounting solution, HistomountTM (National Diagnostics, Atlanta, Georgia) and a coverslip.

2.3.1.4 Microscopy and photography

Equipment

The slides were observed using Axioskop 2 plus (Zeiss) microscope at 20X and 40X.

Photography

Slides were photographed using the NanoZoomer-XR (Hamamatsu) and processed using the NDP.view2 (Hamamatsu) image viewing software.
2.3.2 Polymerase Chain Reaction

2.3.2.1 RNA Extraction

RNA was extracted from fresh or frozen tissue samples from each of the GTKO and GMP pigs using cold RNA Stat-60\textsuperscript{TM} (Tel-Test, Inc., Friendswood, TX, USA) as per manufacturer’s instructions.

Frozen samples were maintained on dry ice before extraction. To extract RNA 100-500 mg of frozen tissue was pulverised in liquid nitrogen using a mortar- and- pestle. The frozen tissue powder was added to a pre-chilled tube containing 2 ml RNA Stat-60\textsuperscript{TM} and homogenized (Power Gen 500 Fischer Scientific) until completely disrupted. The homogenizer was rinsed in between samples with 50 mL RNAse free water (Gibco), 10 ml RNA Stat-60\textsuperscript{TM} and 50 mL RNAse free water (Gibco) to prevent contamination between samples.

After homogenization, 0.4 mL of chloroform (Sigma-Aldrich Laboratories, 372978) was added to each sample, vortexed for 1 minute, allowed to rest for 5 minutes at room temperature, and centrifuged at 4500 rpm in Alegra 15X Centrifuge for 30 min at 4. The aqueous supernatant containing the RNA was collected and transferred to a fresh tube and 1 mL of isopropanol was added to precipitate the RNA. Samples were again centrifuged at 4500 rpm in Alegra 15 X Centrifuge at 4\textdegree C for 20 minutes to recover RNA. Solution was decanted and the pellet was washed with 2 ml of 75\% ethanol by vortexing it and subsequently centrifuging for 10 minutes. The solution was decanted again, and RNA was dissolved in 0.4 mL of RNAse free water (Gibco). The RNA was allowed to dissolve completely and ODs of 260 and 280 nm were measured and recorded using a spectrophotometer (Nanodrop 2000: ThermoScientific). The samples were then frozen in -80\textdegree C until analysed.
2.3.2.2 Reverse Transcriptase

A cDNA was produced using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). For each reverse transcriptase reaction 500 ng of RNA in 10 μL of water was mixed with 10 μL of a 2X RT Master Mix. The master mix consisted of RT buffer, nucleotides, random primers, water and reverse transcriptase enzyme as indicated in Table 2-2. Equal volumes of 2X master mix and RNA (10 μL + 10 μL) were mixed and placed in a thermal cycler. The thermal cycler (Master cycler® personal, Eppendorf) was programmed to the conditions given in Table 2-3. After the reaction, the cDNA was stored at -20°C until used in the PCR amplification. Negative control sample contained 10 μL of PK15 RNA and all components of 2X RT Master Mix except the MultiScribë™ Transcriptase Enzyme.

Table 2-2: 2X RT Master Mix Components: solution used for reverse transcription.

<table>
<thead>
<tr>
<th>Components of 2X RT Master Mix</th>
<th>Volume/Reaction (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x RT Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>25 x dNTP Mix</td>
<td>0.8</td>
</tr>
<tr>
<td>10 x RT R Primers</td>
<td>2.0</td>
</tr>
<tr>
<td>Nuclease free H₂O</td>
<td>4.2</td>
</tr>
<tr>
<td>MultiScribë Transcriptase</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

Table 2-3: The thermal cycler programmed conditions used for Reverse Transcription

<table>
<thead>
<tr>
<th></th>
<th>Step 1 (Anneal)</th>
<th>Step 2 (Extend)</th>
<th>Step 3 (Denature)</th>
<th>Step 4 (Hold)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temp (°C)</strong></td>
<td>25</td>
<td>37</td>
<td>85</td>
<td>4</td>
</tr>
<tr>
<td><strong>Time (min)</strong></td>
<td>10</td>
<td>120</td>
<td>5</td>
<td>(Hold)</td>
</tr>
</tbody>
</table>
2.3.2.3 Polymerase Chain reaction

The cDNA samples underwent a polymerase chain reaction using GoTaq® G2 Green Master Mix (M782A, Promega WI USA) and primers made by Eurofins Genomics (Lot 15-2251).

One set of samples were tested to look for expression of $B4GALNT2$ and another set was used to measure expression of $Beta-Actin (ACTB)$ gene (positive control). Each gene had its own set of forward and reverse primers.

The sequence (5’ to 3’) were as follows:

B4GALNT2 (F):  GCG ACT CCA AAG AAT TGG CTT C
B4GALNT2 (R):  TGG TGA CCT ATG ATC ACG TGT G

B4GALNT2 (F):  TAC AGC CCT AGA TGT CTG TC
B4GALNT2 (R):  CTC TCC TCT GAA AGT GTT CGA G

ACTB (F):  CAA GAT CAT CGC GCC TCC A
ACTB (R):  ACT CCT GCT TGC TGA TCC ACA TCT

The first set of B4GALNT2 primers anneals with exon 10 (forward) and exon 11 (reverse). The amplified product for this set of primers is 110 bp. The second set of B4GALNT2 primers anneals with exon 2 (forward) and exon 3 (reverse) and the amplified product for this set of primers is 331 bp. Both forward and reverse ACTB primers anneal with exon 6 of the gene and the size of the product is 108 bp.
The primers were re-suspended in water to achieve a stock concentration of 100 μMol and the stocks further diluted to make 10 μMol of working primers.

For each PCR reaction, 1 μL of 10 μMol of each forward and reverse B4T primers were added to 5 μL of each of the cDNA samples (containing 125 ng cDNA) and the volume brought to make a total of 12.5 μL by adding nuclease free water. Since ACTB is expressed in large amounts, 37.5 ng of cDNA was used. Therefore, 1 μL of each forward and reverse ACTB primers were added to 1.5 μL of each cDNA sample and the volume brought up to make a total of 12.5 μL by adding nuclease free water. 12.5 μL of GoTaq® G2 Green master mix was then added to each sample to make a total of a 25 μL reaction/sample.

The thermal cycler (Mastercycler® personal, Eppendorf) was programmed to the conditions given in Table 2-4.

<table>
<thead>
<tr>
<th>Step 1 (Denature)</th>
<th>Step 2 (Anneal)</th>
<th>Step 3 (Extend)</th>
<th>Step 4 (Extend)</th>
<th>Step 2 to Step 4 repeated 30 times</th>
<th>Step 5 (Extend)</th>
<th>Step 6 (Hold)</th>
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<tr>
<td>Temp (°C)</td>
<td>95</td>
<td>94</td>
<td>60</td>
<td>72</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td>Time (sec)</td>
<td>300</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>300</td>
<td>(Hold)</td>
</tr>
</tbody>
</table>

2.3.2.4 Gel Electrophoresis

PCR products were separated by gel electrophoresis using 1.5% agarose gels. A 1.5% agarose gel was made using 1.5 g of agarose in 100 mL of 1X TBE Buffer. 1X TBE was made by adding 100 mL of 10X TBE (108 g Tris base, 55 g boric acid and 40 mL 0.5 M EDTA (pH 8.0) in a final volume of 1L of water) to 900 mL of water.
DNA ladder (Promega WI USA) was used as a molecular weight buffer and 15 μL of each sample were ran in a Mini Plus Submarine Gel box (FHU10 Fisher Scientific) for 45 min under 75 W using a PowerPac™ Basic.

2.3.3 FACS analysis of PK15 cells

Pig kidney cells (cell-line 15) (PK15) cells were grown in a 5% CO₂ incubator at 37°C using Dulbecco’s modified Eagles medium DMEM (4.5g/L glucose, Invitrogen, ThermoFischer Scientific) supplemented with 10% fetal bovine serum and non-essential amino acids (Invitrogen, ThermoFischer Scientific).

PK15 cells were collected by gentle trypsin digest, centrifuged at 500 xg, re-suspended on PBS, centrifuged again, re-suspended in DPBS with 1% BSA at 4 x 106/mL and stored on ice. Expression of Sda was measured using a biotin conjugated DBA lectin (5μg/ml, Vector labs) diluted in DPBS with 1% BSA. For staining 50 μL of lectin and 50 μL of cells (200,000 / stain) are mixed in FACs tubes (Falcon™, BD Biosciences, US) and incubated on ice for 45 minutes. Cells were washed with 2 mL of DPBS, centrifuged at 500 xg for 10 minutes, re-suspended in 100 μL of DPBS with 1% BSA containing 0.5 μg/mL of AlexaFluor488 conjugated streptavidin (Invitrogen, ThermoFischer Scientific) and incubated at 4°C for 30 minutes. After staining the cells were again washed with DPBS, re-suspended in 300 μL of PBS with 0.5% formalin and analysed on a FACSVerse™ flow cytometer (BD Biosciences, US). Negative control cells were stained with streptavidin only.
Chapter 3

Results
3.1 **Gene Ontology**

3.1.1 **Prioritisation of proteins for annotation**

Two reviews of aortic valve development and disease provided a comprehensive list of the key genes required for the normal development of an aortic valve (Bonachea et al., 2014, Wu et al., 2017). This list of key genes, along with the 46 gene products associated with the GO term ‘*heart valve development*’ (or its child terms), were combined to provide a primary list of genes that are known to play a role in valve development. Using this information, a secondary list of genes was created to identify genes specifically involved in aortic valve development as that was the focal aim of the project. In order to develop a good understanding of the various processes that play a role in normal heart valve development, I selected genes from different pathways to focus on, including Notch, TGF-beta, and Wnt signaling pathways, and other signaling pathways involved in aortic valve development (Table 3-1).

3.1.2 **Identification of published articles to annotate**

All of the papers listed in the two reviews of aortic valve development and disease (Bonachea et al., 2014, Wu et al., 2017) were screened to identify articles with experimental evidence that would support curation of the prioritised genes. Of the 27 articles initially identified, 18 were selected, as several papers provided overlapping information about the same genes and, therefore, would not have provided additional information (Figure 23). The selected articles were manually curated to create annotations that were submitted to the GO annotation database. Full annotation of the selected papers led to the annotation of additional proteins, 16 which were not in the initial priority list. A few of the genes that had previously been associated with the GO
term ‘heart valve development’ (or child terms) were studied again, and more detailed annotations provided.

![Flowchart](image)

**Figure 23: A flowchart describing the process of selecting papers for curation**

### 3.1.3 Summary of the Gene Ontology annotations created

For the Gene Ontology (GO) section of this project, 378 new GO annotations were associated with 34 protein records (Table 3-1) (Link in references (Ahmed, 2019)). 226 of these annotations were supported by experimental data and 152 annotations were associated with human genes based on structural similarity between the animal (mouse/rat) gene and human gene. The published experimental literature available on the subject of genetic pathways involved in aortic valve disease is mostly based on mouse models, and therefore, the majority of the annotations with experimental evidence codes were associated with mouse proteins rather than human proteins. The murine annotations were then used to infer functionality of human proteins and were
transferred to the human protein records using the ISS-curator evidence code (Inferred by Sequence Similarity, see 2.1.2.4 Evidence Codes) (Primmer et al., 2013).

This project mainly focused on Biological Process GO terms, with occasional annotations made using the Molecular Function and Cellular Component ontology terms. Due to the tiered organization of GO terms within the ontology, specific child terms are found under broader parent terms (Figure 25). Consequently, a thorough search of the GO browser, QuickGO was undertaken to find the most specific and appropriate GO terms. Only these specific GO terms were selected for the inclusion in the annotations. This approach is the most efficient way to annotate but does not lead to a loss of information. For example, ‘aortic valve morphogenesis’ (GO:0003180) is a child term of ‘aortic valve development’ (GO:0003176) which in turn is a child term of ‘heart valve development’ (GO:0003170). If an annotation is made using the term ‘aortic valve morphogenesis’ then this annotation will be retrieved, when the database is searched for any of the three terms listed above.

Although, this project was mainly focused on annotating the genes involved in aortic valve development, comprehensive annotation of each paper, resulted in annotations to other valve development terms. This approach allows for more comprehensive annotations to be created, for each protein, which increases the utility of the GO database as an in-depth and descriptive resource with a breadth of coverage at both gene and knowledge levels. In order to measure the degree of annotation enrichment we calculated the number of genes annotated to valve development (and aortic valve morphogenesis) before beginning and after completion of this project. At commencement of the project, there were 8 annotations using GO terms describing aortic valve development and morphogenesis, associated with 6 different human protein records. This project has added 125 aortic valve development annotations to
28 human proteins, 25 of which were not previously associated with aortic valve development. Of these 125 annotations, 32 are ‘direct’ annotations, and 83 are associated annotation extensions. A direct annotation refers to the primary GO term used to create the basic annotation. However, often, the annotation created does not suffice on its own, and requires contextual information. In such cases, the ‘annotation extension’ field (Figure 24) allows for the curator to add additional data such as anatomical location (specified by Uberon database) or a regulatory target such as expression of a specific gene and thus refine the GO term used (Huntley and Lovering, 2017). Below are 2 examples of annotations with their extensions.

Figure 24: Primary annotations of murine Nos3 and Notch1 proteins with their respective annotation extension fields.

Primary annotations are annotations made to a primary GO term. However, often, the annotation does not provide enough information on its own, and therefore, the Annotation Extension field is used. This field allows for additional data to be added to the primary annotation to make it more elaborate and comprehensive.

Figure 26 below shows the 33 protein records that are associated with ‘aortic valve development’ as of January 2019. This includes 8 proteins that have previously been curated, 3 of which have been updated to reflect additional annotations created as part
of this project. In addition to the 28 proteins curated during this project to aortic valve development, 6 proteins not directly involved in the development of the aortic valve have been curated. 4 of these proteins have been associated with the more general GO term ‘heart valve development’ rather than the aortic valve term, and 2 protein records have shown to have a role in the development of aortic valve disease rather than its morphogenesis. As the GO database is limited to normal processes, annotations to their role in disease could not be made.
Figure 25: Directed Acyclic Graph (DAG) representing the hierarchical relationships of some of the various GO terms used in this project.

All the terms above the other are ancestor terms which will retrieve annotations to its child terms as well. Note how some terms have multiple parents. The black arrows joining a term to ancestor terms indicate an ‘Is a’ relationship, i.e. the child is a subtype of the process described by the parent. The blue arrows indicate the child term is a ‘Part of’ the parent term. The orange, green and red arrows pointing to a term indicates a regulatory relationship, in which the child regulates the process described by the parent. These nested relationships continue back until eventually one will find ‘biological process’ as the root term. At the top of each GO term box is the unique GO identifier (GO ID).
Figure 26: Impact of project on the number of proteins associated.

This figure shows the number of proteins (33) annotated to Aortic Valve Development as of January 2019. 28 of these protein records were annotated to aortic valve development in this project, which included 3 proteins (in green) that have previously been curated and have been updated to reflect additional annotations created as part of this project. 5 protein records (in yellow) were annotated previously, but not curated in this project.
The proteins prioritized for annotation by this project have different roles in valve development. Some proteins are involved in the structural configuration of the valve, while others are involved in early heart development signaling pathways, such as the ‘Notch’, ‘TGF-beta’ and ‘Wnt’ signaling pathways or associated with signaling pathways involved in angiogenesis.

Table 3-1: Human proteins prioritized for annotation in this project.

The proteins are listed according to their primary role in aortic valve development. The HGNC symbol, ID and name are provided, along with the chromosomal location and the UniProt ID. Proteins marked with an asterisk (*) have been annotated previously and only ISS annotations were created for them as part of this project.

<table>
<thead>
<tr>
<th>Structural Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGNC symbol and ID</td>
</tr>
<tr>
<td>1. CILP (HGNC:1980)</td>
</tr>
<tr>
<td>2. ELN (HGNC:3327)</td>
</tr>
<tr>
<td>3. EMILIN1 (HGNC:19880)</td>
</tr>
<tr>
<td>4. MATR3* (HGNC:6912)</td>
</tr>
<tr>
<td></td>
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<tr>
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</tr>
<tr>
<td>5.</td>
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<tr>
<td>6.</td>
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<td>7.</td>
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<td>8.</td>
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<tr>
<td>9.</td>
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<tr>
<td>10.</td>
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<tr>
<td>11.</td>
</tr>
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<td>---</td>
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<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>14</td>
</tr>
</tbody>
</table>

Proteins involved in TGF-beta signaling pathway

<table>
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<tr>
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<th>HGNC name</th>
<th>Chromosomal location</th>
<th>UniProt ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>BMPR2* (HGNC:1078)</td>
<td>bone morphogenetic receptor type 2</td>
<td>2q33.1-q33.2</td>
<td>Q13873</td>
</tr>
<tr>
<td>16</td>
<td>GATA4 (HGNC:4173)</td>
<td>GATA binding protein 4</td>
<td>8p23.1</td>
<td>P43694</td>
</tr>
<tr>
<td>17</td>
<td>GATA5 (HGNC:15802)</td>
<td>GATA binding protein 5</td>
<td>20q13.33</td>
<td>Q9BWX5</td>
</tr>
<tr>
<td>18</td>
<td>SMAD4* (HGNC:6770)</td>
<td>SMAD family member 4</td>
<td>18q21.2</td>
<td>Q13485</td>
</tr>
<tr>
<td></td>
<td>HGNC symbol and ID</td>
<td>HGNC name</td>
<td>Chromosomal location</td>
<td>UniProt ID</td>
</tr>
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</tr>
<tr>
<td>19.</td>
<td>SMAD6 (HGNC:6772)</td>
<td>SMAD family member 6</td>
<td>15q22.31</td>
<td>O43541</td>
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<tr>
<td>20.</td>
<td>SOX9 (HGNC:11204)</td>
<td>SRY-box 9</td>
<td>17q24.3</td>
<td>P48436</td>
</tr>
<tr>
<td>21.</td>
<td>TGFB1 (HGNC:11766)</td>
<td>transforming growth factor beta 1</td>
<td>19q13.1</td>
<td>P01137</td>
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<tr>
<td>22.</td>
<td>ZFPM2* (HGNC:16700)</td>
<td>zing finger protein, FOG family member 2</td>
<td>8q23</td>
<td>Q8WW38</td>
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</table>

Proteins involved in Wnt signaling pathway

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<th>UniProt ID</th>
</tr>
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<td>23. ROCK1 (HGNC:10251)</td>
<td>Rho associated coiled-coil containing protein kinase 1</td>
<td>18q11.1</td>
<td>Q13464</td>
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<tr>
<td>24. ROCK2 (HGNC:10252)</td>
<td>Rho associated coiled-coil containing protein kinase 2</td>
<td>2p25.1</td>
<td>O75116</td>
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</table>

Proteins belonging to other signaling pathways involved in angiogenesis
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<tr>
<th></th>
<th>HGNC symbol and ID</th>
<th>HGNC name</th>
<th>Chromosomal location</th>
<th>UniProt ID</th>
</tr>
</thead>
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<tr>
<td>25.</td>
<td>HECTD1* (HGNC:20157)</td>
<td>HECT domain E3 ubiquitin protein ligase 1</td>
<td>14q12</td>
<td>Q9ULT8</td>
</tr>
<tr>
<td>26.</td>
<td>IGF1 (HGNC:5464)</td>
<td>insulin like growth factor 1</td>
<td>12q23.2</td>
<td>P05019</td>
</tr>
<tr>
<td>27.</td>
<td>MDM4* (HGNC:6974)</td>
<td>MDM4, p53 regulator</td>
<td>1q32.1</td>
<td>O15151</td>
</tr>
<tr>
<td>28.</td>
<td>NFATC1* (HGNC:7775)</td>
<td>nuclear factor of activated T cells 1</td>
<td>18q23</td>
<td>O95644</td>
</tr>
<tr>
<td>29.</td>
<td>PDE2A* (HGNC:8777)</td>
<td>phosphodiesterase 2A</td>
<td>11q13.4</td>
<td>O00408</td>
</tr>
<tr>
<td>30.</td>
<td>RB1 (HGNC:9884)</td>
<td>RB transcriptional corepressor 1</td>
<td>13q14.2</td>
<td>P06400</td>
</tr>
<tr>
<td>31.</td>
<td>ROBO1 (HGNC:10249)</td>
<td>roundabout guidance receptor 1</td>
<td>3p12.3</td>
<td>Q9Y6N7</td>
</tr>
<tr>
<td>32.</td>
<td>ROBO2 (HGNC:10250)</td>
<td>roundabout guidance receptor 2</td>
<td>3p12.3</td>
<td>Q9HCK4</td>
</tr>
</tbody>
</table>
3.1.4 Structural proteins involved in valve development

Heart valves are made of organized extracellular matrix and valve interstitial and endothelial cells (Hinton and Yutzey, 2011). There are a number of proteins that play an important structural role in the formation of the extracellular matrix and composition of the heart valve. A deficiency of these key proteins would be predicted to lead to anomalous composition and malformed valves. In order to understand how these, and other, structural proteins play a role in normal heart valve development, I annotated three proteins, CILP, ELN and EMILIN1 (Table 3-1) and discovered that these three structural proteins also play a role in valve morphogenesis by regulating various signaling pathways.
3.1.4.1 Cartilage Intermediate Layer Protein

Cardiac extracellular matrix (ECM) remodeling is seen as a compensatory mechanism in human heart diseases such as aortic stenosis. The ECM is mainly composed of fibrillar collagen, and various other structural and non-structural proteins; one of these structural proteins is cartilage intermediate layer protein (CILP). Previous research has demonstrated that CILP is involved in cartilage degenerative diseases and plays a role in the inhibition of TGF-beta mediated induction of ECM genes (Seki et al., 2005). A later study also detected CILP in human cardiac muscle tissue and in porcine ECM following ischemia or reperfusion injury (Barallobre-Barreiro et al., 2012). Van Nieuwenhoven et al., 2017 investigated the effect of cardiac disease on CILP expression and test the significance of CILP as a mediator of cardiac ECM remodeling in human and murine models. The study showed that CILP negatively regulated the expression of the Connective Tissue Growth Factor (CTGF) and the alpha-smooth muscle actin (ACTA2) genes in cardiac ventricle fibroblasts, as a part of cellular response to the TGF-beta stimulus. Van Nieuwenhoven et al., 2017 also demonstrated that CILP negatively regulates SMAD protein signal transduction by inhibiting intracellular signaling SMAD proteins, as part of the cardiac ventricle fibroblasts response to TGF-beta. The impact of CILP on the cellular response to TGF-beta was captured using several GO terms and, by exploiting the annotation extension field, CILP was associated with the GO term ‘negative regulation of gene expression’ with the contextual information: regulates expression of ACTA2 and CTGF in cardiac ventricle fibroblasts as part of the cellular response to TGF-beta stimulus. Although, CILP did not appear to have a role in aortic valve development, by regulating expression of genes required for normal valve development, CILP acted as a mediator
for ECM remodeling, hence, suggesting its role in disease progression in patients with aortic valve disease (Van Nieuwenhoven et al., 2017).

### 3.1.4.2 Elastin

The Elastin (ELN) gene encodes for the protein, tropoelastin (Table 3-1). Crosslinking of multiple copies of tropoelastin form the mature protein ‘elastin’, which is one of the major components of elastic fibers. Elastic fibers give structural support to organs and tissues, such as blood vessels, heart, skin lungs and ligaments (Brooke et al., 2003). A study conducted on patients with William Syndrome (WS) - a syndrome with a myriad of manifestations, including supravalvular aortic stenosis (SVAS) - found deletion in an entire elastin locus (Ewart et al., 1994). This led to the hypothesis that, an entire deletion of this gene was associated with WS, but mutations involving part of the ELN gene were responsible for SVAS alone. DNA genomic sequencings of ELN in 2 patients of 1 family, suffering from SVAS, revealed that both affected members of the family had a 100kb deletion of the 3’ end of the gene. The same mutation was seen in another unrelated family that had members suffering from SVAS. DNA sequence analysis localized the deletion between ELN exons 27 and 28. This provided evidence that ELN contains critical exons that have a role in outflow tract and aortic valve morphogenesis. The identified phenotype associated with ELN deletions supported the association of the GO term ‘outflow tract morphogenesis’ with ELN protein record, with the Inferred from Mutant Phenotype (IMP) evidence code (see 2.1.2.4 Evidence Codes).

### 3.1.4.3 Elastin microfibril interface 1

Aortic valve disease is characterized by elastin fiber fragmentation, fibrosis and atypical angiogenesis. Elastin microfibril interface 1 (EMILIN1) is a gene that encodes
the elastin-binding glycoprotein, EMILIN1, that regulates elastogenesis, by stabilizing molecular interactions between elastic fiber components and microfibrils (Zanetti et al., 2004). There had been previous studies that had shown that Emilin1 is required for normal elastic fiber assembly and that this protein, like ELN, inhibits TGF-beta signaling. These studies were able to identify that EMILIN1 inhibits this signaling pathway by binding specifically to the pro-TGF-beta precursor and preventing its maturation in the extracellular space (Zacchigna et al., 2006, Zanetti et al., 2004).

Munjal et al., 2014, investigated the hypothesis that EMILIN1 deficiency in humans can result in AVD, by using a mouse model. Using histology, immunohistochemistry, electron microscopy, quantitative gene expression analysis, immunoblotting and echocardiography, they examined the effects of Emilin1 deficiency in aortic valve tissue, in living Emilin1 knockout mice (Emilin1<sup>−/−</sup>), at juvenile, adult and aged stages (Munjal et al., 2014). Emilin1 was shown to be involved in both, aortic valve morphogenesis, through its role in the regulation of extracellular matrix assembly and collagen fibril organization, and, prevention of aortic valve disease, through downregulation of inflammatory cells, negative regulation of angiogenesis, and downregulation of the TGF-beta signaling pathway. Knockdown of Emilin1 in these mice demonstrated that murine Emilin1 positively regulated the expression of three key aortic valve leaflet ECM structural genes, Chondromodulin (Cnmd), Collagen-type XVIII alpha 1 chain (Col18a1) and Fibulin 5 (Fbln5). Furthermore, Emilin1 downregulated genes such as Matrix metallopeptidase 2 (Mmp2) gene and ‘Elastase, Neutrophil Expressed’ (Elane) gene, which were responsible for cleaving collagen in the ECM and hydrolyzing ECM proteins, respectively. In addition to the regulation of these genes, Emilin1 also was involved in elastic fiber assembly. These processes, all
contributed to the assembly of the ECM, essential for proper aortic valve morphogenesis (Munjal et al., 2014).

Deficiency of Emilin1 in these mice led to development of AVD, resulting in elastic fiber fragmentation, fibrotic aortic valve tissue which progressed to latent AVD, as confirmed by echo, and eventually, premature death (Munjal et al., 2014). Emilin1 -/- mice showed increased fibroblasts and myofibroblast, and an influx of macrophages, which implied the role of Emilin1 in preventing inflammation and fibrosis of the tissue. Furthermore, knockdown of Emilin1 in these mice showed the role that Emilin1 played in downregulation of angiogenesis by negatively regulating several genes, including, Vascular endothelial growth factor a (Vegfa), and Fms Related Tyrosine Kinase 1 (Flt1), as part of the negative regulation of the vascular endothelial growth factor (VEGF) receptor singling pathway. Moreover, Emilin1 -/- mice showed progressive phosphorylated Erk1/2 activation accompanied by increased circulatory plasma levels of active TGF-beta. This highlighted the role of Emilin1 in down regulation of non-canonical phosphorylated Erk1/2 activation, as part of the regulation of TGF-beta signaling. Taken together, these findings suggested that human EMILIN1 was likely to have a role in the prevention of the AVD process (Munjal et al., 2014).

Curation of this paper led to the association of GO terms, such as ‘aortic valve morphogenesis’, ‘positive regulation of extracellular matrix assembly’, ‘negative regulation of transforming growth factor beta receptor signaling pathway’ and ‘positive regulation of gene expression’, amongst others, with Emilin1, using the IMP evidence code (see 2.1.2.4 Evidence Codes). Following confirmation using the HCOP tool, that the mouse Emilin1 gene and the human EMILIN1 gene were orthologs, the valve development-relevant annotations associated with the mouse Emilin1 protein were transferred to the human EMILIN1 protein record using the ISS-Curator evidence
code (see 2.1.2.4 Evidence Codes). These annotations summarize the evidence provided in this paper that murine Emilin1 played a role in aortic valve development and prevention of the development of AVD.

3.1.5 Signaling pathways involved in valve development

![Signaling pathways involved in valve development](image)

Figure 27: The coregulation of various signaling pathways that are involved in heart valve development and remodeling.

The signaling network demonstrates the numerous pathways and transcriptional regulators that act in a coordinated manner to regulate the process of heart valve formation. Each signaling pathway shown is indicated by the key protein the pathway is named after, thus providing a simplified schema of the signaling events that occur. Red arrows denote positive/synergistic interactions between pathways. Blunt red arrows denote inhibitory effects between pathways (reproduced from Ehrin J. Armstrong, and Joyce Bischoff Circulation Res. 2004;95:459-470).
Three of the signaling pathways required for healthy aortic valve development (Figure 27), NOTCH, TGF-beta, and WNT, have been curated, in addition to proteins that are part of other signaling pathways involved in angiogenesis. The full set of annotations created as part of this thesis can be retrieved online using the link listed in the references (Ahmed, 2019). These pathways were selected because of their role in early valve development. The annotation of each protein is discussed below, in general, starting with the ligand and ending with the downstream effectors.

3.1.5.1 NOTCH signaling pathways associated with valve development

![NOTCH signaling pathway diagram](image)

Figure 28: NOTCH signaling pathway.

The NOTCH receptor is a tri-domain receptor consisting of the Notch Trans-Membrane (TM) domain, the Notch ExtraCellular Domain (NECD), and the Notch IntraCellular Domain (NICD). A Notch ligand (DLL4/JAG1-2) binds to the NOTCH receptor, which leads to S2 cleavage of the NOTCH receptor (mediated by TACE), and the separation of the NECD (still bound to ligand) from the other two domains. This is followed by another cleavage (S3, by gamma secretase) which separated the NICD from the TM domain. NICD then translocates to the nucleus where it binds to RBPJ and initiates transcription of target genes such as HEY2 and SNAI1.
The Notch signaling pathway (Figure 28) has been found to play a major role in the cardiac development, with direct roles in valve development (Niessen et al., 2008, Aquila et al., 2013). The roles of some of the proteins involved in valve development-relevant Notch signaling pathways have been captured, by this thesis, using GO terms, through the annotation of a wide range of published literature and are explained below.

Proteins present in the Notch signaling pathway

DLL1, DLL3, DLL4*, JAG1*, JAG2, NOTCH1*, RBPJ*, HEY1*, HEY2*, HEYL*, SNAIL1*, SNAIL2*

All proteins marked with an asterisk (*) have been annotated as part of this project, with an addition of NOS3, which although, not part of the signaling pathway directly, has been shown in this thesis to positively regulate NOTCH1. The remaining proteins of the signaling pathway were not annotated, as there was no data associated with them in the papers selected for curation.

Figure 29: A comparison of the GO annotations associated with the key proteins involved in the NOTCH signaling pathways done prior to and after completion of this project.
The network was created using Cytoscape and the associated GO terms were overlaid using the GOlorize and BinGO apps (see methods). Each node represents a protein, each edge represents an interaction captured in the interaction databases used. The colored segments of the nodes represent the GO terms associated with each protein, see below the networks for a key to the GO term colors. The post-project Cytoscape analysis shows that all (except 2) of the key proteins in the pathway are now associated with the GO term ‘aortic valve development’, or one of its child terms. The pink circle around NOTCH1 represents the fact that it is included in the Genomics England PanelApp list of genes involved in aortic valve disease.

**Delta like canonical Notch ligand 4**

Delta like canonical Notch ligand 4 (DLL4), is a Notch activator (ligand) encoded by the *DLL4* gene. The close relationship between the Notch and TNF signaling pathways was investigated by Wang et al., 2017, who used DLL4 as the ligand to stimulate these pathways in mouse models of arterial valve development and showed that human DLL4 positively regulated expression of murine *Caspase 3* (*Casp3*), *Caspase 8* (*Casp8*), *Tumor necrosis factor* (*Tnf*) and, *TNF superfamily member 10*, (*Tnfsf10*) genes in endothelial cells of the mouse arterial valves, during arterial valve morphogenesis (Wang et al., 2017a). The annotation of the experimental data described in Wang et al. led to the creation of 34 annotations to 5 different protein records and included detailed information about the genes regulated by DLL4 and its impact on valve development.

**Jagged 1**

Two papers provided annotations for jagged 1 (JAG1), as well as other participants in the Notch signaling pathway. As described above, the binding of the ligand, JAG1, to the receptor, NOTCH1, triggers a series of proteolytic cleavages that lead to the activation of NOTCH1 target genes. This was demonstrated by McBride et al., 2008, investigating *NOTCH1* mutations, found in patients that exhibited left ventricular outflow tract (LVOT) defects. Equivalent mutations were introduced into a construct encoding rat Notch1 to assess the effects of these mutants on Notch1 function.
Transfection of wild-type rat Notch1 into murine NIH3T3 cells demonstrated a significant increase in Notch1 activity when co-cultured with Ltk-mouse fibroblasts (L-cells) expressing the rat Jag1 ligand, providing experimental evidence, that Jag1 is required to activate the Notch signaling pathway (McBride et al., 2008). An annotation using the term ‘positive regulation of Notch signaling pathway’ was made to the rat Jag1 protein record and subsequently transferred to the human JAG1 protein record using the Inferred from Sequence Similarity (ISS) evidence code (see 2.1.2.4 Evidence Codes).

Riley et al., 2011, continued the study of NOTCH1 missense alleles associated with LVOT defects. Human Microvascular Endothelial Cells (HMECs) transfected with wild type rat Notch constructs and co-cultured with HMECs transfected with rat Jag1, undergo epithelial-to-mesenchymal transition (EMT). Through the linked activity between Jag1 and Notch1, as evident by the co-culture assay, the paper highlighted the role of Jag1 in positively regulating cardiac EMT in microvascular endothelial cells by increasing the expression of key transcription factors involved in EMT; hes related family bHLH transcription factor with YRPW motif proteins (HEY2, HEYL), and Snail family transcriptional repressors (SNAI1, SNAI2) (Riley et al., 2011). This information was captured using the GO terms, ‘positive regulation of gene expression’ (with the regulated genes listed as targets in the annotation extension field) and ‘positive regulation of cardiac epithelial-to-mesenchymal transition’ using the Inferred from Genetic Interaction (IGI) evidence code and including rat Notch1 in the WITH field (see 2.1.2.4 Evidence Codes). Although this experiment was not done in the context of valve morphogenesis, due to the role played by Jag1 in the Notch signaling pathway, and statements by the authors in their paper, an additional annotation of human JAG1 to the term ‘aortic valve morphogenesis’ was made using
the Traceable Author Statement (TAS) evidence code (see 2.1.2.4 Evidence Codes). Experimentally supported annotations made associated with the rat Jag1 protein record were subsequently transferred to the human JAG1 protein record using the ISS-curator evidence codes (see 2.1.2.4 Evidence Codes).

**Notch Receptor 1**

Notch receptor 1 (*NOTCH1*) is one of four members of the Notch family, all of which are involved in a variety of developmental processes by controlling cell fate (Lai, 2004). Due to the widespread role of the Notch signaling pathway and its role in the cardiovascular system, the *Notch1* gene has been one of the most widely studied genes in relation to valve development. A total of six papers were curated to explore the various roles of the *NOTCH1* gene in valve development.

McBride et al., 2008, demonstrated mutations in the *NOTCH1* gene (affecting the Notch signaling pathway) were present in some patients with a range of LVOT defects. Through studying genetic variants found in patients with LVOT malformations, this paper provided Inferred from Mutant Phenotype (IMP) evidence that NOTCH1 was involved in ‘*aortic valve morphogenesis*’ and ‘*outflow tract morphogenesis*’ (McBride et al., 2008).

As mentioned above, Riley et al., 2011, explored the molecular mechanisms that contributed to reduced Notch signaling and impaired development of the LVOT by co-culturing HMECs expressing wild type or mutant rat Notch vectors with HMECs transfected with rat Jag1. These experiments demonstrated that Notch1 positively regulates of expression of key EMT-associated transcription factors (*HEY2, HEYL, SNAI1, and SNAI2*) (Riley et al., 2011). These transcription factors regulate the expression of proteins, such as actin, E-cadherin, fibronectin and cytokeratin that are
responsible for cell adhesion, proteolytic activity, cytoskeletal remodeling (Kim and Lee, 2014, Haynes et al., 2011) key processes required for cardiac EMT. Thus, this data provided more details about the individual molecular events that lead to cardiac EMT. This information was captured using the GO terms, ‘positive regulation of gene expression’ and ‘positive regulation of cardiac epithelial-to-mesenchymal transition’ with rat Notch1 using the IGI evidence code, including rat Jag1 in the WITH field. (see 2.1.2.4 Evidence Codes). This article also provided experimental evidence that Notch1 was located in the plasma membrane (Riley et al., 2011). Annotations made to the rat Notch1 protein record were subsequently transferred to the human NOTCH1 protein record using the ISS-curator evidence codes (see 2.1.2.4 Evidence Codes).

In the third paper annotated, Acharya et al., 2011, inhibited Notch1 signaling by the application of a gamma-secretase inhibitor (the endopeptidase required to release NICD), in the rat aortic valve and demonstrated that this led to low levels of expression of Notch1 downstream targets, increased calcification and reduced ExtraCellular Matrix (ECM) assembly in the aortic valve. As the inhibition of gamma secretase may have inhibited the activity of other proteins it was not possible to annotate the role of Notch based on this experiment. However, by using a Col2a1 luciferase reporter co-transfected with a constitutively active Notch1 NICD and Sox9, Acharya et al., confirmed that, Notch1 via Sox9, positively regulated the transcription of Collagen type II alpha 1 chain (Col2a1). Although, no direct inhibition of calcification was seen by Notch1 in this paper, the loss and gain-of-function studies of Notch downstream targets, Hey1 and Hey2, in a well-established cell culture model for valve calcification, supported the hypothesis that Notch1 inhibited calcification (Acharya et al., 2011).

To confirm the role Notch1 has in calcific AVD, Bosse et al., 2013 transfected porcine aortic valve interstitial cells (PAVICS) with Notch1 NICD and assessed the effect on
calcification by measuring nodule formation and Alizarin red staining and found a significant decrease in calcification in presence of NICD (Bosse et al., 2013). Furthermore, the subcellular fractionation data confirmed the nuclear location of Notch1 NICD in the aortic valve. Bosse et al., also examined the cardiac morphology of Notch1 heterozygote (Notch1 +/-) mice and compound mutant mice (Nos3 +/-; Notch1 +/-). Echocardiography and morphological analysis showed that Notch1 heterozygote mice have low levels of aortic valve calcification. In contrast, the compound mutant mice had a high degree of abnormal aortic valve morphology, and increased Alcian blue staining and smooth muscle actin (SMA) expression, both indicators of aortic valve disease. This data confirmed that Notch1 is involved in aortic valve morphogenesis and prevents calcification of the aortic valve. In addition, the use of these compound Nos3−/−;Notch1+− mice also showed that nitric oxide (NO) regulation of Notch1 signaling leads to an increase in the expression of Sox9 in aortic valve (Bosse et al., 2013).

Using Notch1 +/- heterozygote, and Notch1 +/-; Nos3 +/- compound mutant mice described above, Koenig et. al., 2016, studied the role of endothelial Notch1 in the development of semilunar valves and cardiac outflow tract using mice embryos. Histological analysis and immunofluorescence of hearts obtained from these mice embryos exhibited thickened malformed semilunar (aortic and pulmonary) valves, defective endocardial cushion formation and ventricular septal defects, demonstrating the role that Notch1 with Nos3 had in normal aortic valve morphogenesis, pulmonary valve morphogenesis, endocardial cushion morphogenesis and ventricular septum morphogenesis (Koenig et al., 2016). Annotations to mouse Notch1 were created using the IGI evidence code due to its linked activity with Nos3 (see 2.1.2.4 Evidence Codes). Annotations made to the mouse Notch1 protein record were subsequently
transferred to the human NOTCH1 protein record using the ISS-curator evidence code (see 2.1.2.4 Evidence Codes).

A complete knockout of Notch1 is lethal, consequently, a further insight into the Notch signaling pathway was provided by Wang et al., who, through use of mouse conditional models of Notch1 mutant phenotypes, altered Notch signaling in endothelial or interstitial cells of developing valves (Wang et al., 2017a). Wang et al., demonstrated that inactivation of Notch1 in valvar endothelial cells resulted in a wide range of valvar and myocardial defects including enlarged semilunar valves, fibrotic valves, ventricular septal defects and hypertrophic left ventricle, providing experimental evidence that Notch1 was involved in, ‘aortic valve morphogenesis’, ‘pulmonary valve morphogenesis’, ‘positive regulation of endothelial cell apoptotic process’, ‘negative regulation of cardiac muscle hypertrophy’ and ‘negative regulation of extracellular matrix constituent secretion’ amongst others; all of these processes being part of normal aortic valve and cardiac morphogenesis (Wang et al., 2017a).

**Recombination signal binding protein for immunoglobulin kappa J region**

Recombination signal binding protein for immunoglobulin kappa J region (RBPJ) is an important transcriptional regulator in the Notch signaling pathway. When bound to the Notch NICD in the nucleus, it acts as a transcriptional activator proteins by recruiting chromatin remodeling complexes and regulating the transcription of the Notch target genes (Castel et al., 2013, Borggrefe et al., 2016, Gomez-Lamarca et al., 2018). As RBPJ is the main nuclear partner of the Notch signaling pathway, the mouse Rbpj -/- knockouts have been used to explore the role of the protein on development and homeostasis of arterial valves in an attempt to understand the role of the Notch
signaling pathway in post-EMT development (Wang et al., 2017a). Rbpj -/- mouse embryos have hypertrophic aortic and pulmonary valves along with sub-aortic ventricular septum defects. These defects appear to be due to the reduced expression of two key apoptotic genes, tumor necrosis factor (Tnf) and TNF superfamily member 10 (Tnfsf10) in the post-EMT valves, both of which are positively regulated by Notch1 and Rbpj. Without Tnf and Tnfsf10 the valves become hypertrophic and collagenized, signifying their importance in aortic valve morphogenesis (Wang et al., 2017a). This information was captured using the GO terms ‘aortic valve development’, ‘pulmonary valve development’ and ‘ventricular septum morphogenesis’ and ‘positive regulation of gene expression’ (with Tnf and Tnfsf10 included in the annotation extension) using the IMP evidence code (see 2.1.2.4 Evidence Codes). As above, the annotations made to the mouse Rbpj protein record were subsequently transferred to the human RBPJ protein record using the ISS-curator evidence codes (see 2.1.2.4 Evidence Codes).

Hes related family bHLH transcription factors with YRPW: motif family

The hes related family bHLH transcription factor with YRPW motif family (HEY1, HEY2 and HEYL), are all members of the Hairy and enhancer of split (Hes)-related family of basic helix-loop-helix type transcription factors. All three of the HEY genes are effectors of the Notch signaling pathway. Similar to the Hes proteins they repress target genes by forming nuclear dimers that localize to the nucleus and repress transcription (Weber et al., 2014).

Missense alleles in the human NOTCH1 gene have been linked with a reduction in ligand-induced Notch signaling (McBride et al., 2008). Riley et al., 2011, described above, studied the molecular mechanisms that led to defective epithelial-to-
mesenchymal transition (EMT) associated with these missense alleles, by co-culturing cells obtained mutant rat models expressing rat Notch1 with the same missense alleles, and HMECs, and then measured the levels of the Hey Notch target genes. Riley et al., 2011 showed that co-culturing HMECs with the rat Jag1 ligand expressing cells, induced EMT and that Jag1-dependent expression of Hey2 and Heyl is reduced in the Notch1 mutant cells (Riley et al., 2011). Although the direct roles of Hey2 and HeyL were not explored by Riley et al., 2011, another study, Fischer et al., 2007 (previously annotated by our group) has shown that HEY2 and HEYL represent direct Notch targets that play an important role in cardiac EMT (Fischer et al., 2007). This information has been quoted by Riley et al., based on which, the terms ‘epithelial to mesenchymal transition involved in endocardial cushion formation’, ‘notch signaling involved in heart development’ and ‘aortic valve morphogenesis’ were associated with HEY2 and HEYL using the TAS evidence code (see 2.1.2.4 Evidence Codes).

Curation of another paper, Acharya et al., 2011, exploring the role of Notch signaling in inhibition of calcification, showed that overexpression of mouse Hey1 or Hey2 in PAVICs resulted in a decrease in osteopontin (SPP1: secreted phosphoprotein 1), a protein associated with calcification and bone remodeling. This provided experimental evidence that Hey1 and Hey2 were involved in prevention of calcification and aortic valve disease (Acharya et al., 2011). This information was captured using the GO terms ‘negative regulation of gene expression’ and ‘negative regulation of biomineral tissue development’ using the IDA evidence code. The annotations made to the mouse Hey1 and Hey2 protein records were subsequently transferred to the human HEY1 and HEY2 protein records using the ISS evidence code.
**Snail family transcriptional repressors: 1 and 2**

Snail family transcriptional repressors 1 (SNAI1 or Snail) and 2 (SNAI2 or Snail2) are zinc finger transcriptional repressors which represses the expression of adhesion molecule E-cadherin and claudins with concurrent upregulation of vimentin and fibronectin as part of SNAI1 induced EMT during embryonic development (Kaufhold and Bonavida, 2014). Riley et al., 2011, (see above) noted that LVOT-associated NOTCH1 alleles led to defective EMT through impaired ligand dependent induction of SNAI1 and SNAI2, and proposed that these transcription factors are involved in the regulation of genes required for normal EMT (Riley et al., 2011). Consequently, the terms ‘epithelial to mesenchymal transition involved in endocardial cushion formation’, ‘notch signaling involved in heart development’ and ‘aortic valve morphogenesis’ were associated with both SNAI1 and SNAI2 using the author statement (TAS) evidence code (see 2.1.2.4 Evidence Codes). The experimental evidence to support these annotations was not provided in the Riley et al., 2011 paper, instead the paper referenced another study, previously annotated by our group, which showed that during cardiac EMT, endocardial cells undergo significant changes in gene expression including Notch1-dependent induction of Snai1, and Snai2 (Niessen et al., 2008).

**Nitric Oxide Synthase 3**

Nitric Oxide (NO) is a lipophilic molecule that regulates many vasculature relevant biological processes, including, vascular tone, cellular proliferation and leukocyte adhesion. NO is generated by three synthases: endothelial nitric oxide synthase (eNOS/NOS3), inducible NOS (iNOS/NOS2) and neuronal NOS (nNOS/NOS1) (Bosse et al., 2013). The mature aortic valve is composed of a structural trilaminar
extracellular matrix interspersed with aortic valve interstitial cells and covered by endothelium which express NOS3. Previous studies have shown that NOS3 plays a role in Calcific Aortic Valve Disease (CAVD), but the mechanism was largely unknown. Similarly, increased expression of NOTCH1 has been genetically linked to human CAVD (Garg et al., 2005).

In order to establish whether there was a relation between endothelial NO and NOTCH1 in the development of CAVD, Bosse et al. 2013 co-cultured porcine aortic valve interstitial cells (PAVICs) with NO-secreting human umbilical vein endothelial cells (HUVECs) (Bosse et al., 2013). Using histological staining and immunofluorescence, Bosse et al., measured the effect of endothelial NO (synthesized by NOS3) on calcification. Decreased calcification was seen in PAVICs in the presence of endothelial NO (when co-cultured with HUVECs) compared to absence of HUVECs. Similarly, PAVICs cultured with Nos3+/+ mouse lung endothelial cells (MLECs) demonstrated decreased calcification in comparison to when they were cultured with MLECS obtained from Nos3−/− mice. Furthermore, as mentioned in the NOTCH1 section above, hearts of 6-8-week-old Nos3−/− and Nos3−/−; Notch1+/− mice showed abnormal aortic valve morphology, and increased Alcian blue staining and SMA expression, both indicators of aortic valve disease (Bosse et al., 2013). This provided evidence that Nos3 involved in aortic valve morphogenesis and prevented calcification of the aortic valve. This information was captured using the GO terms ‘aortic valve morphogenesis’ and ‘biomineral tissue development’. Bosse et al., 2013 also showed that endothelial NO positively regulated Notch1 signaling in PAVICs, which was evident by an increased expression of the Notch target proteins, Hey1 when PAVICs were co-cultured with HUVECS or a NO donor.
The understanding of the role of Nos3 was expanded, as described above, through the investigation of the Notch1 +/-, Nos3 -/-, and Notch1 +/+-; Nos3 -/- mice embryos (Koenig et al., 2016). Histology and immunofluorescence analyses of hearts obtained from these mice revealed thickened malformed semilunar valves (aortic and pulmonary) and ventricular septal defects, demonstrating the role that Nos3 had in normal endocardial cushion morphogenesis, aortic valve leaflet morphogenesis, pulmonary valve morphogenesis and ventricular septum morphogenesis. Annotations to mouse Nos3 were created using the IGI evidence code due to its linked activity with Notch1 (see 2.1.2.4 Evidence Codes). Annotations made to the mouse Nos3 protein record were subsequently transferred to the human NOS3 protein record using the ISS-evidence code (see 2.1.2.4 Evidence Codes).

3.1.5.2 The role of TGF-beta signaling in valve development

Figure 30: TGF-beta signaling pathway.
The binding of TGF-beta ligands to TGF-beta receptors initiates the signaling pathway. In the canonical pathway, the ligand-receptor interaction causes phosphorylation of SMAD2 and 3. The phosphorylated SMAD2 and 3 then bind to SMAD4, and the complex translocates to the nucleus and causes transcription of target genes i.e. GATA4 and GATA5. In the non-canonical pathway on the other hand, the same ligand-receptor interactions are involved but, in this case, the dimerized and activated receptor serves as a binding site for signaling molecules, such as TAK1, TAB1, and GRB2. GRB2 interacts with the SOS complex which after a series of other interactions activates ERK. ERK is then responsible for inhibiting GSK3B which in turn inhibits SMAD3 and 4, thereby reducing canonical TGF-beta signaling.

The transforming growth factor-beta (TGF-beta) signaling pathway plays a critical role in the regulation of cell growth, differentiation, and development in a wide range of biological systems (Gordon and Blob, 2008, Frangogiannis, 2017). The pathway includes the, TGF-beta family ligands (TGFB1, TGFB2, TGFB3): the main signal transducers for TGF-beta receptors, SMADs: a family of structurally similar proteins, (SMAD1-9), and the transcription factors which are responsible for regulating the signaling pathway’s target genes (Figure 30) (Miyazono, 2000).

Proteins present in the TGF-beta signaling pathway

TGFB1*, TGFB2, TGFB3, TGFB1, TGFB2, SMAD2, SMAD3, SMAD4, SMAD6*, GATA4*, GATA5*.

All proteins marked with an asterisk (*) have been annotated as part of this project, with an addition of SOX9, which although, not part of the signaling pathway directly, has been showed to be positively regulated by TGFB1. The remaining proteins of the signaling pathway were not annotated, as they have been annotated previously.
Figure 31: A comparison of the GO annotations associated with the key proteins involved in the TGF-beta signaling pathway done prior to and after completion of this project.

The network was created using Cytoscape and the associated GO terms were overlaid using the GOlorize and BinGO apps (see methods section). Each node represents a protein, each edge represents an interaction captured in the interaction databases used. The colored segments of the nodes represent the GO terms associated with each protein, see below the networks for a key to the GO term colors. The post project Cytoscape analysis shows that TGFB1, SMAD6, GATA4, GATA5 and SOX9 of are annotated to aortic valve development. The pink circle around SMAD6 represents the fact that it is included in the Genomics England PanelApp list of genes involved in aortic valve disease.

Transforming Growth Factor Beta 1

Transforming Growth Factor Beta 1 (TGFB1) is a protein of the Transforming Growth Factor-beta (TGF-beta) superfamily of cytokines and is responsible for the regulation of several cellular processes, including, proliferation, differentiation and apoptosis, and heart valve development (Gordon and Blob, 2008, Frangogiannis, 2017). The TGFB1 signaling pathways is one of the several signaling pathways that regulate SOX9 expression and localization. Whilst studying the regulation of SOX9 in calcific aortic valve disease, Huk et al., 2016 investigated the ability of TGFB1 to recapitulate the protective effects of Vascular Endothelial Cells (VECs) on Vascular Interstitial Cell (VIC) mediated calcification (Huk et al., 2016). Endothelial-specific deletion of Tgfb1 in mice models led to increased Sox9 expression, calcific nodule formation, and
aortic valve dysfunction in vivo. This information was captured using GO terms ‘aortic valve morphogenesis’, and, ‘negative regulation of biomineral tissue development’ using the IMP Evidence Code (see 2.1.2.4 Evidence Codes). The annotations made to the mouse Tgfb1 protein record were subsequently transferred to the human TGFB1 protein record using the ISS-curator evidence code (see 2.1.2.4 Evidence Codes). Furthermore, PAVICs treated with media containing human TGFB1 were shown to localize Sox9 to the nucleus, and to increase the expression of COL2A1 and SMAD2 in these cells. The terms ‘positive regulation of pathway-restricted SMAD protein phosphorylation’ and ‘positive regulation of protein localization to nucleus’ were, therefore, associated with TGFB1 using the IDA evidence code.

**GATA binding proteins**

GATA binding protein 4 and 5 (GATA4, 5) are zinc-finger transcription factors that are known to play a critical role in normal aortic valve development. Both GATA4 and GATA5 are linked to the TGF-beta signaling pathways as their transcription is regulated by under the effect of Smad3, a downstream target of TGF-beta (Nagaraj and Datta, 2010). The important role of GATA4 in aortic valve development was confirmed by Li et al., 2018, who screened the coding and non-coding regions of the GATA4 gene by direct sequencing in 150 index patients with congenital bicuspid aortic valve (BAV) and matched it against controls (unaffected family members of an identified mutation carrier and 300 unrelated healthy individuals). Using a dual-luciferase reporter assay system, the functional effect of the mutation was confirmed (Li et al., 2018). Based on the evidence suggesting association of GATA4 loss-of-function mutation to an enhanced susceptibility to BAV, the term ‘aortic valve morphogenesis’ was associated with GATA4 using the evidence code, IMP (see 2.1.2.4 Evidence Codes). Furthermore, the paper also provided evidence that GATA4
positively regulates the transcription of NOS3 (which plays a role in valve development: see section 3.1.5.1), which provided additional confirmation that GATA4 is involved in valve development.

Similar to GATA4, the importance of GATA5 in normal aortic valve development has been demonstrated by the association of GATA5 mutations with BAV (Laforest et al., 2011, Padang et al., 2012, Shi et al., 2014). Padang et al. identified the presence of four rare non-synonymous variations within the GATA5 transcriptional activation domains in a patient, two of which, Gln3Arg and Tyr142His substitutions, were at functionally conserved residues and therefore likely to impact on the transcriptional activation of GATA5 target regions. Similarly, Shi et al. genotyped GATA5 in patients with BAV, and using a luciferase reporter assay system, characterized the functional effect of the mutations, which led to identification of two novel heterozygous mutations which were associated with loss of function of GATA5. These papers provided evidence that variations in GATA5 transcriptional activation domains may play a role in development of BAV in humans. Using the information provided by both papers, the GO term ‘aortic valve morphogenesis’ was associated with GATA5, using the evidence code, IMP (see 2.1.2.4 Evidence Codes).

Further annotations were associated with human GATA5 based on mouse model data. Laforest et al., 2011, demonstrated that the targeted deletion of the mouse Gata5 led to hypoplastic hearts and partially penetrant BAV formation. In addition, endocardial cell-specific inactivation of Gata5 led to BAV, similar to that observed in the Gata5-/ mice (Laforest et al., 2011). These data were used to support the association of the GO terms, ‘aortic valve morphogenesis’, ‘negative regulation of left ventricular hypertrophy’, ‘negative regulation of cardiac muscle hypertrophy’ and ‘positive regulation of cardiac endothelial to mesenchymal transition’, using the IMP evidence
code (see 2.1.2.4 Evidence Codes). The latter three annotations associated with the mouse Gata5 protein record were subsequently transferred to the human GATA5 protein record using the ISS-curator evidence code (see 2.1.2.4 Evidence Codes).

The endocardial cell-specific inactivation of murine Gata5 suggested that the role of GATA5 in human aortic valve morphogenesis might be due to defective endocardial cell differentiation, resulting from the deregulation of various components of the Notch pathway and other important endocardial cell regulators. Laforest et al., showed that murine Gata5 is required for normal endocardial cushion fusion, (captured using the GO term ‘endocardial cushion fusion’). Additional contextual information was included in the GO annotations associated with Gata5 to capture its role, as a DNA binding transcription factor, positively regulating expression of, bone morphogenetic protein 4 (Bmp4), cadherin 5 (Cdh5), EPH receptor B4 (Ephb4), erb-b2 receptor tyrosine kinase 2 (Erbb2), Hey1, Jag1, myocyte enhancer factor 2C (Mef2c), Notch1, neuregulin 1 (Nrg1), T-box 20 (Tbx20), and TEK receptor tyrosine kinase (Tek), as part of aortic valve morphogenesis. Based on the patterns of gene expression seen in this mouse model, Gata5 was associated to the negative regulation of expression of the following genes; natriuretic peptide A (Nppa), natriuretic peptide B (Nppb) and Rbpj. The increase in levels of Rbpj seen in Gata5 −/− embryos is indicative of the dysregulation of the Notch Pathway found in the absence of Gata5, as Rbpj is a downstream effector of Notch Signaling (see section 3.1.2.1) and acts as a transcriptional repressor in the absence of Notch activation. These results indicated that Gata5 is an important regulator of genes involved in endothelial cell differentiation, and that the expression of Gata5 in endothelial cells is required for proper development of the endocardial cushion and positive regulation of cardiac endothelial to mesenchymal transition in outflow tract and atrioventricular canal in
embryo. Moreover, the data confirmed that, absence of *Gata5* resulted in defective valve morphogenesis and BAV formation (Laforest et al., 2011).

**SMAD family**

Several members of the SMAD family are involved in the TGF-beta signaling pathways, however, to date variants in only SMAD6 have been associated with aortic valve disease. SMAD6 is an inhibitory SMAD that regulates TGF-beta signaling pathways by competing with SMAD4 for binding with receptor-activated SMAD1, thereby preventing the transcription of SMAD4 target genes (Hata et al., 1998). In addition to its role in TGF-beta signaling, SMAD6 is also an intracellular inhibitor of the related BMP signaling pathways. As previous studies had shown that BMP signaling is required for normal heart valve and outflow tract development, Tan et al., 2012, studied three genes of the BMP signaling pathway, BMPR1A, BMPR2 and SMAD6, for novel variants in 436 cases of cardiovascular malformations. Two variants of SMAD6 (C484F and P415L), were identified and molecular functional studies confirmed that these variants, when compared to wild type SMAD6, showed reduced (almost complete and partial loss respectively) inhibitory activity (Tan et al., 2012). The patient phenotypic data provided evidence that mutations in SMAD6 protein lead to aortic valve malformations, enabling the association of the GO term, ‘*aortic valve morphogenesis’ with SMAD6 using the evidence code IMP (see 2.1.2.4 Evidence Codes). Immunoblotting and alkaline phosphatase (ALP) assays showed that expression of BMP receptor 1 (BMPR1) and ALP was higher in cells transfected with SMAD6 variants. This data was captured using the GO terms ‘*negative regulation of BMP signaling pathway*’ and ‘*negative regulation of osteoblast differentiation*’.
SRY-box 9

SRY-box 9 (SOX9) is a transcription factor involved in chondrocyte differentiation, and is of interest to this project because it has been found to play a causative role in the onset of calcific AVD (Lincoln et al., 2007, Peacock et al., 2010). Furthermore, the expression of SOX9 is regulated by both TGFB1 and NOTCH1 signaling pathways. As there is strong evidence by Huk et al., 2016, that presence of Tgfb1 increases nuclear localization of Sox9, and additional studies showing the positive regulation of Sox9 expression by TGF-beta (Coricor and Serra, 2016, Chavez et al., 2017), I have chosen to include Sox9 as a part of the TGF-beta signaling pathway.

VICs within the aortic valve leaflets differentiate toward an osteoblast-like cell and deposit bone-like matrix that leads to leaflet stiffening and calcific aortic valve stenosis (Hinton et al., 2006, Lincoln et al., 2007, Rajamannan et al., 2011). Since, SOX9 is highly expressed in VICs, Huk et al., 2016, explored the mechanisms responsible for regulation of SOX9 in human, porcine and murine models of CAVD, and found that nuclear localization of Sox9 is reduced in CAVD. Along with annotations for 3 other proteins (link in references (Ahmed, 2019)), this paper provided evidence that human SOX9 was a nuclear component of the endothelial cell (Huk et al., 2016).

Due to the established role of Notch signaling pathway in development of CAVD, Acharya et al. studied the changes in gene expressions that occur by inhibition of the Notch signaling pathways in rat AVICs, and found significant down regulation of Sox9, and cartilage specific genes such as Col2a1. The COL2A1 gene is responsible for producing type II collagen chains that are important structural components of the aortic valve (Peacock et al., 2010). Acharya et al., 2011, investigated the role of Sox9 and Notch1 in the regulation of Col2a1 by measurement of luciferase activity in mouse
COS7 cells transfected with Sox9 and NICD plasmids. Results showed increase in Col2a1 luciferase activity in presence of Sox9. In addition, the paper provided experimental evidence through histological staining that Sox9 significantly reduced calcification in PAVICs and also reversed the calcification associated with Notch Inhibition in these cells (Acharya et al., 2011). This information was captured using the GO term ‘negative regulation of biomineral tissue development’ using the contextual information ‘aortic valve’ and part of ‘aortic valve morphogenesis’ using the IDA evidence code (see 2.1.2.4 Evidence Codes).

### 3.1.5.3 The role of Wnt signaling in valve development

As described in the introduction, there are three major Wnt (Wingless/iNTegrated) signaling pathways, all of which are involved in heart valve development (Alfieri et al., 2010, Zhang et al., 2015), with the noncanonical pathway especially playing a role in aortic valve calcification (Albanese et al., 2017).

In the planar cell polarity noncanonical pathway, Wnt signaling is transduced through frizzled via the interaction of the PDZ and DEP domains of DVL and the small GTPase Rho, ROCK is activated (Figure 32) (Katoh and Katoh, 2007, Komiya and Habas, 2008).

**Proteins present in the Planar Cell Polarity WNT signaling pathway**

WNT, FZD, DVL, DAAM1, RHO, ROCK*, RAC, JNK, AP-1

The only two proteins annotated from this signaling pathway were ROCK1 and ROCK2. The role of the remaining proteins involved in the various Wnt signaling pathways in aortic valve development is yet to be annotated.
Figure 32: Planar Cell Polarity (PCP) non-canonical WNT signaling pathway.

In the PCP non-canonical pathway, the WNT-FZD complex activates the Disheveled protein (DVL) and through DAAM1 mediates activation of RHOA and RAC which cause activation of ROCK and JNK subsequently. JNK1 is then translocated to the nucleus and binds to AP-1 causing transcription of target genes.

Figure 33: A comparison of the GO annotations associated with the key proteins involved in the WNT signaling pathway done prior to and after completion of this project.
The network was created using Cytoscape and the associated GO terms were overlaid using the GOlorize and BinGO apps (see Methods section). Each node represents a protein, each edge represents an interaction captured in the interaction databases used. The colored segments of the nodes represent the GO terms associated with each protein, see below the networks for a key to the GO term colors. The post project Cytoscape analysis shows that ROCK1 and ROCK2 have been annotated to heart valve and aortic valve development for the first time in this project.

**Rho associated coiled-coil containing protein kinases: 1 and 2**

The highly coordinated action of the different signaling pathways in the regulation of heart valve development is demonstrated by the role of Rho associated coiled-coil containing protein kinase: 1 (ROCK1) and 2 (ROCK2). These proteins act as major downstream effectors of Planar Cell Polarity (PCP) non-canonical Wnt signaling pathway, as well as functioning downstream of TGFB1. (Hartmann et al., 2015, Julian and Olson, 2014). Previous studies have shown that ROCK functions downstream of TGFB1, consequently Huk et al., investigated the role of Rock1 and Rock2 in Tgfb1-mediated Sox9 nuclear localization. A ROCK inhibitor was used to treat porcine AVICs and then calcification was measured.

Furthermore, calcification was increased in samples where the ROCK inhibitor was used which indicated that ROCK1 and ROCK2 were involved in Sox9 nuclear localization and prevented calcification (Huk et al., 2016). Based on this experimental data, the GO term ‘positive regulation of protein localization to nucleus’ was associated with both porcine ROCK1 and ROCK2, with the annotation extension capturing that Sox9 was being located to the nucleus and that this process was part of the ‘cellular response to transforming growth factor beta stimulus’. In addition, the term ‘negative regulation of biomineral tissue development’, as part of ‘aortic valve morphogenesis’ was also associated with these two proteins, in this and the annotation above the IGI evidence code was applied (see 2.1.2.4 Evidence Codes).
3.1.5.4 Additional proteins with a role in aortic valve development

There are many additional proteins that play a role in aortic valve development. I have annotated 7 of these genes.

**RB transcriptional corepressor 1**

RB transcriptional corepressor 1, RB1, formerly called retinoblastoma 1, encodes the retinoblastoma protein (pRb). pRB is a multifunctional protein that has many binding and phosphorylation sites. The pRb is responsible for maintenance of mesenchymal cell differentiation as well as bone development and soft tissue calcification. As VICs are mesenchymal in nature, and diseased human valves have a tendency to calcify, Freytsis et al., 2018, investigated the possible role of the retinoblastoma protein (pRb) in aortic valve disease. Using echocardiography, histology and immunohistochemistry amongst other imaging techniques, they examined the effects of pRB deficiency in aortic valve tissue, in conditional Rb1 knockout mice (pRB cKO) in the aortic valve, regulated by Tie2-Cre-mediated excision of floxed RB1 alleles. pRb cKO animals showed significantly more aortic valve regurgitation by echocardiography compared to pRb heterozygous control animals (Freytsis et al., 2018). The pRb cKO aortic valves had increased leaflet thickness without increased cellular proliferation. Further histologic studies demonstrated intense ACTA2 expression in pRb cKO leaflets associated with disorganized extracellular matrix and increased leaflet stiffness. Furthermore, the pRb cKO mice also showed increased circulating cytokine levels. Based on the results provided, annotations were made to ‘aortic valve morphogenesis’, ‘negative regulation of inflammatory response’, ‘negative regulation of myofibroblast differentiation’, ‘positive regulation of extracellular matrix organization’, and ‘positive regulation of collagen fibril organization’ using the IMP evidence code. Following confirmation using the HCOP tool, that the mouse Rbl gene and the human
*RB1* gene were orthologs, the valve development-relevant annotations associated with the mouse *Rbl* gene were transferred to the human *RB1* gene using the ISS-Curator evidence code.

**Slit Guidance Ligands: 2 and 3**

Slit guidance ligands: 2 (SLIT2) and 3 (SLIT3) are extracellular proteins that bind roundabout guidance receptors (ROBO1 and 2) to initiate the Slit-Robo signaling pathway. SLIT2 and 3 serve as guidance molecules for axon and are involved in organogenesis and angiogenesis. These proteins, like their receptors (ROBO1 and 2), are expressed in or adjacent to all cardiac cushions and valves. Mommersteeg et al., 2015, studied *Slit2* /− and *Slit3* /− mice and found membranous ventricular septum defects. Since the membranous interventricular septum and the atrioventricular and semi-lunar valves develop from primitive myocardial cushions lining the tube, annotations associated with *Slit2* and *Slit3* were made to ‘*aortic valve morphogenesis*’, ‘*pulmonary valve morphogenesis*’, and ‘*ventricular septum morphogenesis*’, using the IMP evidence code (Mommersteeg et al., 2015). Following confirmation using the HCOP tool, that the mouse *Slit2* and *Slit3* genes and the human *SLIT2* and *SLIT3* genes are orthologs, the valve development-relevant annotations associated with the mouse *Slit2* and *Slit3* protein records were transferred to the human *SLIT2* and *SLIT3* protein records using the ISS-Curator evidence code (see 2.1.2.4 Evidence Codes).

**Roundabout Guidance Receptors: 1 and 2**

Roundabout guidance receptors: 1 (ROBO1) and 2 (ROBO2) are transmembrane proteins that serve as axon and cell adhesion receptors. Previous studies on animal models have shown that the Roundabout signaling pathway is involved in cell adhesion during cardiac cell polarization, cardiac neural crest migration and adhesion,
Ahmed, 2019

and cardiac chamber formation (Santiago-Martínez et al., 2008, Morin-Poulard et al., 2016). Mommersteeg et al., previously found expression of Robo1 and Robo2 receptors in or adjacent to all cardiac cushions and valves (Mommersteeg et al., 2013). Based on this finding, Mommersteeg et al. investigated the effects of knocking out Robo and its ligand Slit on cardiac development (Mommersteeg et al., 2015). Using various tools including immunohistochemistry, valve and ventricular length and volume measurements and luciferase assays, Mommersteeg et al., provided experimental evidence that loss of Robo1 or both Robo1 and Robo2 resulted in membranous ventricular septum defects, thickened immature semilunar and atrioventricular valves, and bicuspid aortic valves. This information was captured using the terms ‘aorta development’, ‘aortic valve morphogenesis’, ‘endocardial cushion formation’, ‘outflow tract septum morphogenesis’, ‘pulmonary valve morphogenesis’, and ‘ventricular septum morphogenesis’, using the IGI evidence code (see 2.1.2.4 Evidence Codes).

It has also been proposed that the Robo-Slit signaling pathway may act by regulating the Notch1 signaling pathway (Mommersteeg et al., 2015). An increase in expression of Notch1 and its downstream gene targets, Hey1 and Heyl, was seen in Robo1+/+ hearts when compared to Robo1−/−. In addition, co-transfection of Robo1 or Robo2 with NICD resulted in further increase in luciferase activity by 23-fold. These results highlighted a role of Robo1 and 2 in regulation of the Notch signaling pathway and the data was captured by associating the GO term, ‘positive regulation of Notch signaling pathway involved in heart induction’, using the IDA evidence code, with both Robo1 and Robo2.

Following confirmation using the HCOP tool, that the mouse Robol and Robo2 genes and the human ROBO1 and ROBO2 genes are orthologs, respectively, the valve
development-relevant annotations associated with the mouse Robo proteins were transferred to the human ROBO protein record using the ISS-curator evidence code (see 2.1.2.4 Evidence Codes).

**Tumor necrosis factor receptor superfamily members: 1A and 1B**

Tumor necrosis factor receptor superfamily members 1A (*TNFRSF1A, alias TNFR1*) gene, and 1B (*TNFRSF1B, alias TNFR2*) gene, encode the proteins, TNFRSF1A and TNFRSF1B, respectively. TNFRSF1A and TNFRSF1B are membrane receptors that bind tumor necrosis factor (TNF). These receptors can activate transcription factors, mediate apoptosis, and also act as a regulator of inflammation (Li and Anderson, 2018, Parameswaran and Patial, 2010). This superfamily is also known to regulate the activity of other receptor signaling pathways (Dempsey et al., 2003), and that their ligands are not all TNF superfamily members. Previous studies have shown that nerve growth factor receptor, NGFR, another member of the TNFRSF family, acts as a receptor for non-TNF superfamily ligands, such as nerve growth factor (NGF) (Twohig et al., 2011), and that NGF binding to NGFR activates and regulates Notch (Salama-Cohen et al., 2005). Furthermore, while exploring the role of the Notch signaling pathway in the development of arterial valves, Wang et al., 2017, identified a Notch1-Tnf signaling axis that regulated post-EMT development of arterial valves. This discovery was achieved by altering Notch signaling in endothelial or interstitial cells of developing valves by the use of *Notch1* /- and *Rbpj* /- mice (see section 3.1.5.1). Quantitative PCR analysis showed increased expression of apoptotic genes, such as *Tnf*, in the presence of NICD, and decreased expression in both *Notch1* /- and *Rbpj* /- mice (Wang et al., 2017a). Furthermore, deleting both *Tnfrsf1a* and *Tnfrsf1b* receptors in mice led to development of hypertrophic arterial valves with increased collagen deposition. This led to the conclusion that Tnf-regulated apoptosis is
necessary for development of arterial valves and suggested that it is also involved in postnatal valvar calcification. The \textit{Tnfrsf1a} and \textit{Tnfrsf1b} experimental data presented by Wang et al., was captured by associating the terms ‘\textit{aortic valve development}’, ‘\textit{pulmonary valve development}’, ‘\textit{negative regulation of extracellular matrix constituent secretion}’, ‘\textit{negative regulation of cardiac muscle hypertrophy}’, and ‘\textit{positive regulation of apoptotic process involved in morphogenesis}’ with these protein records, along with the IGI evidence code (see 2.1.2.4 Evidence Codes). The annotation extension information that this occur in both aortic and pulmonary valves was added to the last of these 3 terms. Following confirmation using the HCOP tool, that the mouse \textit{Tnfrsf1a} and \textit{Tnfrsf1b} genes and the human \textit{TNFRSF1A} and \textit{TNFRSF1B} genes are orthologs, respectively, the valve development-relevant annotations associated with the mouse \textit{Tnfrsf1a} and \textit{Tnfsf1b} protein records were transferred to the human \textit{TNFRSF1A} and \textit{TNFRSF1B} protein records using the ISS-Curator evidence code.

\textbf{Cytoscape Analysis}

Using Cytoscape analysis as described in the methods, a graphic network of protein-protein interactions was created using the following proteins (to which annotations were made in this thesis) as seeds: BMPR2, CILP, DLL4, ELN, EMILIN1, GATA4, GATA5, HECTD1, HEY1, HEY2, HEYL, IGF1, JAG1, MATR3, MDM4, NFATC1, NOS3, NOTCH1, PDE2A, RB1, RBPJ, ROBO1, ROBO2, ROCK1, ROCK2, SLI2, SLIT3, SMAD4, SMAD6, SNAI1, SNAI2, SOX9, TGFB1, TNFRSF1A, TNFRSF1B, ZFPM2, TBX5. This network included 1514 protein interactions (edges) provided by MINT, IntAct and EBI-GOA databases, leading to a network with 1318 proteins (nodes). Using GOlorize and BiNGO, the network was overlaid with the GO terms enriched in the network, using the GO annotation file available at the start of the
project at the start of the project and at the end of the project (Figure 34). Table 3-2 lists 10 GO terms enriched in the analysis of the whole network which were most relevant to this project, with aortic valve development being enriched multiple folds after inclusion of annotations created in this project. The $p$-value of the term ‘aortic valve development’ within the GO dataset pre project was 4.07E-03 which after completion of the project, became much more significant, at 1.65E-32, demonstrating the impact of improving the annotation of proteins involved in this process, by carrying out this project. All terms apart from one, regulation of Wnt signaling pathways, were enriched and $p$-values became more significant for all of them. The GO terms associated with these proteins are now a much more accurate reflection of the current state of understanding about the roles of various signaling pathways and proteins involved in the aortic valve development.
Figure 34: The impact of this project on the interpretation of the aortic valve development interactome.

The GO terms associated with the proteins annotated in this project and their interacting proteins, in February 2017 (before the start of this project) compared to the GO terms associated with this network in March 2019 (at the completion of this project). The network was created using Cytoscape and the associated GO terms were overlaid using the GOlorize and BinGO apps. Each node represents a protein, each edge represents an interaction captured in the interaction databases used. The colored segments of the nodes represent the GO terms associated with each protein, see below the networks for a key to the GO term colors. The post project Cytoscape analysis shows that all proteins annotated as part of this project (highlighted with a red circle) apart from three have now been annotated to at least one of the three GO terms representing valvar development. The three proteins listed in the Genomics England PanelApp as involved in aortic valve disease, are indicated by a pink circle around the nodes (ELN, NOTCH1 and SMAD6) all three of which have been annotated in this project. The proteins circled in blue represent the protein records not annotated to at least one of these three valvar terms as part of previous curation efforts.
Table 3-2: Comparison of 10 GO terms most relevant to this project prior to and after the annotations submitted in this project.

The table shows the enrichment of the 10 GO terms most relevant to this project. All terms have significant $p$-values; GO terms with $p$-values <0.01 identified on Cytoscape are considered as significant. 9/10 terms were enriched, with the largest degree of enrichment seen for the term ‘aortic valve development’ after the project’s completion. Study n refers to the number of proteins in the network that have annotations to the corresponding GO term and the Population n refers to the number of proteins in the human proteome with annotations associated with the GO term. Although 1318 proteins were analyzed from the network, only 1085 were associated with any GO terms.

<table>
<thead>
<tr>
<th>GO term</th>
<th>GO dataset post project (2019)</th>
<th>GO dataset pre project (2018)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p$-value</td>
<td>Study (n=1085)</td>
</tr>
<tr>
<td>heart valve development</td>
<td>1.37E-35</td>
<td>41</td>
</tr>
<tr>
<td>aortic valve development</td>
<td>1.65E-32</td>
<td>29</td>
</tr>
<tr>
<td>semi-lunar valve development</td>
<td>2.28E-32</td>
<td>31</td>
</tr>
<tr>
<td>heart development</td>
<td>2.08E-31</td>
<td>108</td>
</tr>
<tr>
<td>regulation of transforming growth factor beta receptor signaling pathway</td>
<td>2.95E-26</td>
<td>45</td>
</tr>
<tr>
<td>cardiac ventricle development</td>
<td>3.56E-23</td>
<td>45</td>
</tr>
<tr>
<td>cardiac septum development</td>
<td>3.17E-21</td>
<td>40</td>
</tr>
<tr>
<td>endocardial cushion development development</td>
<td>6.72E-21</td>
<td>26</td>
</tr>
<tr>
<td>regulation of Wnt signaling pathway</td>
<td>5.67E-18</td>
<td>68</td>
</tr>
<tr>
<td>regulation of Notch signaling pathway</td>
<td>1.66E-17</td>
<td>34</td>
</tr>
</tbody>
</table>
3.2 Cardiac Morphology

In order to understand the development of aortic valve disease, fetal to adult specimens were examined and classified according to the morphological variants of aortic valve, the site of fusion and the component of the raphae. In addition, it was important to be able to take accurate measurements of this structure during its development. Consequently, a variety of methodologies were investigated to identify the most reliable measuring methods, as well as examining the feasibility of applying these methods on specimens from fetal to adults, with or without a diagnosis of aortic valve stenosis. In addition, long term storage of specimens leads to distortion of the tissue, therefore, new approaches to fix the specimens were investigated.

3.2.1 Gross Morphology

3.2.1.1 Demographics

Initially, it was necessary to classify the specimens according to aortic valve type. There are a total of 86 specimens diagnosed as having ‘aortic valve stenosis’ held under license in the ICS Cardiac Archive. After applying the exclusion criteria, 56 of the specimens were selected for examination which included, 41 Unicuspid Aortic Valve (UAV), 14 Bicuspid Aortic Valve (BAV) and 1 Tricuspid Aortic Valve (TAV) specimen(s).
Figure 35: Graphical representation of the demographics of specimens selected for gross morphological examination

The flowchart describes the sequence of the selection of specimens, and the number of specimens which had their age and sex documented. The left pie chart represents the number of different morphological variants of aortic stenosis samples seen: 41 unileaflet Aortic Valves (UAV), 14 bileaflet aortic valves (BAV), and 1 trileaflet aortic valve (TAV). The right pie chart shows the total number of specimens belonging to each age group: 43/56 specimens belonged to the fetal/pediatric age group and 13/56 specimens belonged to the adult age group.

Of the 56 specimens, 43 belonged to the fetal and pediatric age group, whereas, 13 were adult specimens. 43 of the total specimens examined had post mortem papers
available. Out of these 43, 41 had their sex documented, and 30 had their age documented.

3.2.1.2 Prevalence of morphological variants in different age groups

83% of specimens that belonged to the fetal and pediatric age group had UAV, and the remaining 27% exhibited BAV. However, majority of adult specimens had BAV, with only 38% having UAV (Figure 36).

![Flowchart describing the prevalence of morphological variants in different age groups.](image)

Using information provided by the post mortem papers where available, a breakdown of the cases was plotted according to specific age groups. All of the 6 fetal specimens exhibited UAV, however, the majority of the UAV cases belonged to the neonate (0-1 month) age group. The least prevalent age group was ‘>3m – 6m’ and ‘>1y -6y’ (Figure 37). The adult age groups ranged from 18-80y, with 54% suffering from BAV (Figure 38).
Figure 37: Prevalence of unileaflet aortic valves (UAV) and bileaflet aortic valves (BAV) in fetal-pediatric age groups.

This graph represents the prevalence of UAV shown in green and the BAV shown in blue. There were 6 fetal samples with the rest all post-partum samples. However, the majority of samples belonged to the neonatal age group (0-1 month). Overall, cases of UAV were much more prevalent than the BAV cases in this age group.
Figure 38: Prevalence of unileaflet aortic valves (UAV) and bileaflet aortic valves (BAV) in adult age groups.

This graph represents the prevalence of UAV shown in green and the BAV shown in blue. The majority of the cases didn’t have their age specified. The ones that did ranged from 18 to 80 years. Out of 12 adult samples, 5 were UAV with the majority exhibiting BAV.
3.2.1.3 Prevalence of morphological variants in both sexes

The ratio of UAV to BAV was 3:2 in females, whereas, in male cases, the ratio of UAV:BAV was slightly higher, 3:1 (Figure 39)

![Prevalence of morphological variants of AVD in both sexes](image)

**Figure 39: Prevalence of morphological variants of AVD in both sexes.**

The graph above represents the prevalence of UAV shown in green and the BAV shown in blue in both sexes. 76% of the male samples were UAV, and 24% were BAV. In female samples, the percentage of UAV at 60% was less than that of males, with 40 % exhibiting BAV.

3.2.1.4 Phenotypes of UAV

There were 3 phenotypes of the UAV, depending upon sites of fusion of the leaflets. The most common variant found in 83% of the samples, had sites of fusion between, the Left Leaflet (LL) and Right Leaflet (RL), and, the RL and the Non-Coronary Leaflet (NCL). The other 2 variants with fusion between, RL-NCL-LL, and NCL-LL-RL were seen in 12% and 5% of the samples, respectively (Figure 40). The 95% Confidence Interval (CI) calculated for each phenotype is shown in Table 3-3.
### Table 3-3: Confidence Interval for phenotypes of UAV

<table>
<thead>
<tr>
<th>Phenotype of UAV</th>
<th>Proportion (%)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-RL and RL-NCL</td>
<td>83</td>
<td>69-91</td>
</tr>
<tr>
<td>RL-NCL and NCL-LL</td>
<td>12</td>
<td>5-26</td>
</tr>
<tr>
<td>NCL-LL and LL-RCL</td>
<td>5</td>
<td>1-16</td>
</tr>
</tbody>
</table>

**Figure 40: Prevalence of phenotypes of UAV.**

The most common phenotype (83%) were fusions between, left leaflet (LL) and right leaflet (RL), and RL and non-coronary leaflet (NCL). The other phenotypes with fusions between RL-NCL and NCL-LL, and fusions between NCL-LL and LL-RCL accounted for 12% and 5% of the samples respectively.
3.2.1.5 Phenotypes of BAV

There were 3 phenotypes of the BAV, as determined by the site of fusion of adjacent leaflets. The most common variant found in 57% of the samples, had fusion between the LL and the RL. The next most common variant, seen in 36% of the samples, had fusion between RL-NCL. The least common variant seen in 1 sample (7%) had fusion between NCL-LL (Figure 41). The 95% CI calculated for each morphological variant is shown in Table 3-4.

<table>
<thead>
<tr>
<th>Phenotype of BAV</th>
<th>Proportion (%)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-RL</td>
<td>57</td>
<td>33-79</td>
</tr>
<tr>
<td>RL-NCL</td>
<td>36</td>
<td>16-61</td>
</tr>
<tr>
<td>NCL-LL</td>
<td>7</td>
<td>1-31</td>
</tr>
</tbody>
</table>

Figure 41: Prevalence of phenotypes of BAV.

The two more prevalent phenotypes found were fusion between, left leaflet (LL) and right leaflet (RL) at 57%, and fusion between RL and non-corporal leaflet (NCL) at 36%. Fusion between NCL-LL was only seen in 1 sample.
3.2.1.6 Component of Raphe

Raphe formed at the site of fusion of leaflets could either be, fibrous, valvar or, have both fibrous and valvar components. In the fetal and pediatric age group, majority of the raphe found were ‘fibrous’ or ‘fibro-valvar’. The assessment carried out showed, 39 L-R raphe (25 fibrous, 13 fibro-valvar, and 1 valvar), 38 R-N raphe, (29 fibrous, and 9 fibro-valvar), and lastly 7 N-L raphe (4 fibrous, 2 fibro-valvar, and 1 valvar). In adult samples, however, none of the raphe present were entirely fibrous, but were either, fibro-valvar or valvar. Assessment of the raphe showed, 7 L-R raphe (3 fibro-valvar and 4 valvar), 8 R-N raphe (3 fibro-valvar and 5 valvar), and 1 N-L raphe which was valvar (Figures 42, 55 and 60). The 95% CI calculated for each raphe is shown in Table 3-5.
Figure 42: Comparison of the prevalence of each type of raphe between fetal and pediatric (FP) samples and samples belonging to the adult (A) age group and their site of fusion.

In FP group, majority of the raphe found were ‘fibrous’ or ‘fibro-valvar’. Raphe formed between right leaflet (RL) and non-coronary leaflet (NCL) were found to be most fibrous, followed by raphe between Left-Leaflet (LL) and RL. Raphe between NCL-LL had the lowest ration of Fibrous raphe, albeit still totaling to 57%, and also had 14% of raphe that were valvar in nature. In adult samples, however, none of the raphe present were entirely fibrous, but were either, fibro-valvar or valvar. Assessment of the raphe showed, 7 L-R raphe (3 fibro-valvar and 4 valvar), 8 R-N raphe (3 fibro-valvar and 5 valvar), and 1 N-L raphe which was valvar.
### Table 3-5: Confidence Intervals for Proportion of Raphe

<table>
<thead>
<tr>
<th>LL and RL raphe</th>
<th>Fetal/Pediatrics</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion (%)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Fibrous</td>
<td>64</td>
<td>48-77</td>
</tr>
<tr>
<td>Fibro-valvar</td>
<td>33</td>
<td>20-49</td>
</tr>
<tr>
<td>Valvar</td>
<td>3</td>
<td>0.5-13</td>
</tr>
<tr>
<td>Fibrous</td>
<td>76</td>
<td>60-87</td>
</tr>
<tr>
<td>Fibro-valvar</td>
<td>24</td>
<td>13-39</td>
</tr>
<tr>
<td>Valvar</td>
<td>0</td>
<td>0-9</td>
</tr>
<tr>
<td>Fibrous</td>
<td>57</td>
<td>25-84</td>
</tr>
<tr>
<td>Fibro-valvar</td>
<td>29</td>
<td>8-64</td>
</tr>
<tr>
<td>Valvar</td>
<td>14</td>
<td>3-51</td>
</tr>
</tbody>
</table>
3.2.1.7 Correlation between Area of Interleaflet Triangles and Type of Raphe

There were significant differences between the means of the interleaflet area in cases where there were no fusion of leaflets and in leaflets that were fused. Furthermore, if the raphe was fibrous or fibro-valvar the area of interleaflet triangle was noted to further decrease (Figures 43 and 57). A similar trend was seen in adult samples, although, none of the raphe were entirely fibrous (Figures 44 and 52).

The mean, SD, and median of each type of raphe are shown in Tables 3-6 and 3-7. One-way ANOVA analysis was performed to compare whether presence of raphe, and the type of raphe had a significant effect on the area of interleaflet triangles.

<table>
<thead>
<tr>
<th>Type of Fusion</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>q1</th>
<th>q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Fusion</td>
<td>41</td>
<td>0.36</td>
<td>± 0.28</td>
<td>0.28</td>
<td>0.17</td>
<td>0.52</td>
</tr>
<tr>
<td>Valvar Raphe</td>
<td>2</td>
<td>0.03</td>
<td>± 0.03</td>
<td>0.03</td>
<td>0.0</td>
<td>0.06</td>
</tr>
<tr>
<td>Fibro-valvar Raphe</td>
<td>24</td>
<td>0.06</td>
<td>± 0.16</td>
<td>0</td>
<td>0.0</td>
<td>0.04</td>
</tr>
<tr>
<td>Fibrous Raphe</td>
<td>57</td>
<td>0.02</td>
<td>± 0.10</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

ANOVA analysis of fetal and pediatric samples showed $f$-ratio value = 27.0 and a $p$-value < 0.00001 indicating that the results were significant.
Table 3-7: Statistical Analyses of Type of Fusion in adult samples

<table>
<thead>
<tr>
<th>Type of Fusion</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>q1</th>
<th>q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Fusion</td>
<td>19</td>
<td>0.30</td>
<td>± 0.18</td>
<td>0.24</td>
<td>0.85</td>
<td>0.43</td>
</tr>
<tr>
<td>Valvar Raphe</td>
<td>11</td>
<td>0.06</td>
<td>± 0.08</td>
<td>0.02</td>
<td>0.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Fibro-valvar Raphe</td>
<td>7</td>
<td>0.06</td>
<td>± 0.06</td>
<td>0.04</td>
<td>0.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

ANOVA analysis of adult samples showed $f$-ratio value = 14.0 and a $p$-value of <0.000039 indicating that the results were significant.
Figure 43: Graphical representation of the correlation of the area of interleaflet triangle and the type of raphe in fetal and pediatric samples.

The ratio (area of the interleaflet triangle: cross-sectional area of the aorta) was calculated to standardise the area of interleaflet triangle according to the size of the specimens. Comparison of the ratio shows a significant reduction in the mean and median in cases where there was no fusion to cases where there was raphe present. Furthermore, fibrous component of the raphe shows an even further decrease than its valvar counterparts.
The ratio (area of the interleaflet triangle: cross-sectional area of the aorta) was calculated to standardise the area of interleaflet triangle according to the size of the specimens. Comparison of the ratio shows a significant reduction in the mean and median in cases where there was no fusion to cases where there was raphe present.

Figure 44: Graphical representation of the correlation of the area of interleaflet triangle and the type of raphe in adult samples.
3.2.1.8 Valve Dysplasia

28/43 (65\%) of the fetal and pediatric age group samples showed no calcification, whereas the remaining 15 (35\%) samples had nodularity present on the leaflets (Figures 45 and 58). In the adult age group, 6/13 (46\%) samples had no calcification, 1/13 (8\%) had nodularity, and the remaining 6/13 (46\%) showed calcification, ranging from mild-severe (Figures 46 and 53).

Figure 45: Valve leaflet anatomy in fetal and pediatric samples.

28/43 (65\%) of the fetal and pediatric age group samples showed no calcification, nor exhibited any other nodularity or dysplasia. The remaining 15 (35\%) samples had nodularity present on the leaflets.
Figure 46: Valve leaflet anatomy in adult samples.

In the adult age group, 6/13 (46%) samples had no calcification, 1/13 (8%) had nodularity, and the remaining 6/13 (46%) showed calcification, ranging from mild to severe.

Table 3-8: Confidence Interval of Morphology of Aortic Valve leaflets

<table>
<thead>
<tr>
<th>Morphology of Aortic Valve Leaflets</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal/Pediatrics</td>
</tr>
<tr>
<td>No Calcification</td>
<td>50-78</td>
</tr>
<tr>
<td>Dysplastic Valve</td>
<td>22-50</td>
</tr>
<tr>
<td>Mild Calcification</td>
<td>-</td>
</tr>
<tr>
<td>Moderate Calcification</td>
<td>-</td>
</tr>
<tr>
<td>Severe Calcification</td>
<td>-</td>
</tr>
</tbody>
</table>

3.2.1.9  Fibrosis/ Endocardial FibroElastosis in Valve Phenotypes

Fibrosis/Endocardial FibroElastosis (EFE) was classified as ‘none-mild’ and ‘moderate-extensive’. 74% of fetal and pediatric samples with UAV exhibited moderate-extensive fibrosis compared to 40% of adult samples with UAV. Similarly, 57% of pediatric samples with BAV exhibited moderate-extensive fibrosis, with 43%
having none-mild fibrosis, compared to adult samples, where all the BAV samples had none-mild fibrosis (Figures 47 and 54, Table 3-9).

Table 3-9: Confidence Interval for severity of fibrosis seen in unileaflet and bileaflet aortic valves

<table>
<thead>
<tr>
<th>Fibrosis/EFE in UAV</th>
<th>95% Confidence Interval</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal/Pediatrics</td>
<td>Adults</td>
</tr>
<tr>
<td>None-Mild</td>
<td>12-38</td>
<td>23-88</td>
</tr>
<tr>
<td>Moderate-Extensive</td>
<td>62-88</td>
<td>12-77</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fibrosis/EFE in BAV</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal/Pediatrics</td>
</tr>
<tr>
<td>None-Mild</td>
<td>16-75</td>
</tr>
<tr>
<td>Moderate-Extensive</td>
<td>25-84</td>
</tr>
</tbody>
</table>
Figure 47: Prevalence of EFE in different valve phenotypes.

Comparison of Fibrosis/Endocardial FibroElastosis (EFE) between fetal and pediatric (P) specimens show that 74% of fetal/pediatric samples with UAV (Unicuspid) exhibited moderate-extensive fibrosis compared to 40% of adult (A) samples with UAV. Similarly, 57% of Pediatric samples with BAV (Bicuspid) exhibited moderate-extensive fibrosis, with 43% had none-mild fibrosis, compared to adult samples, where all the samples had none-mild fibrosis.
3.2.1.10 Correlation of site of fusion with progression of fibrosis/EFE

Although, fetal and pediatric sample exhibited a greater degree of EFE than adults, 83% of the fetal and pediatric samples with LL-RL fusion had moderate – extensive EFE as compared to 57% of the fetal and pediatric samples that did not have fusion between LL and RL. In adults, 25% of samples with LL-RL fusion had moderate-extensive EFE, however, there were no samples without LL-RL fusion, so a comparison couldn’t be made (Figure 48, table 3-10).

A z-score test to test significance of both proportions showed the value of \( p \) is 0.12. The result is therefore not significant at \( p < 0.05 \).

Table 3-10: Confidence Interval of correlation of site of fusion with progression of Fibrosis/EFE

<table>
<thead>
<tr>
<th>Fibrosis/EFE in samples with fusion between LL and RL</th>
<th>95% Confidence Interval</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal/Pediatrics</td>
<td>Adults</td>
</tr>
<tr>
<td>None-Mild</td>
<td>10-31</td>
<td>41-93</td>
</tr>
<tr>
<td>Moderate-Extensive</td>
<td>69-90</td>
<td>7-59</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fibrosis/EFE in samples without fusion between LL and RL</th>
<th>95% Confidence Interval</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal/Pediatrics</td>
<td>Adults</td>
</tr>
<tr>
<td>None-Mild</td>
<td>15-75</td>
<td>51-100</td>
</tr>
<tr>
<td>Moderate-Extensive</td>
<td>25-85</td>
<td>0-50</td>
</tr>
</tbody>
</table>
Figure 48: Correlation of site of fusion to progression of EFE in fetal/pediatric and adult samples.

Comparison of the degree of EFE between fetal and pediatric and adult samples showed that fetal and pediatric samples exhibited a greater degree of EFE than adults, however, an increase of 26% in prevalence of moderate-extensive EFE was seen in fetal/pediatric samples with LL-RL fusion as opposed to those that did not have fusion between LL and RL. In adults, 25% of samples with LL-RL fusion had moderate-extensive EFE, however, there were no samples without LL-RL fusion, so a comparison could not be made.
3.2.1.11 LV remodeling in relation to fibrosis/EFE

Left ventricular thickness was measured at the lower 1/3\textsuperscript{rd} from the apex and then analysed as a percentage of the mean length of the ventricular wall, which was calculated by measuring inner and outer lengths of the LV wall (Figure 51). Bland-Altman plots for Intra- and Inter-observer variability showed high reproducibility (Figure 49).

![Bland Altman plots showing intra- and inter-observer variability.](image)

**Figure 49:** Bland Altman plot showing intra- and inter-observer variability.
The average of two measures (x-axis) was plotted against the observer differences between two measures (y-axis). The mean for the intra-observer variability was -0.33 ± 0.78 and for the Inter-observer variability was -0.35 ± 0.84. Both observations showed high reproducibility.

The mean percentage of LV thickness/length in fetal and pediatric samples that had none-mild fibrosis/EFE was 16.7%, as compared to samples that had moderate-extensive EFE who had a mean of 27.1% (Figures 50 and 59).

Table 3-11: Statistical analyses of the association of LV remodeling with degree of Fibrosis/EFE

<table>
<thead>
<tr>
<th>None -Mild Fibrosis/EFE</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>q1</th>
<th>q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal/Pediatric Samples</td>
<td>11</td>
<td>16.7</td>
<td>± 5.4</td>
<td>16</td>
<td>11.8</td>
<td>19.5</td>
</tr>
<tr>
<td>Adult Samples</td>
<td>11</td>
<td>18.7</td>
<td>± 6.4</td>
<td>18</td>
<td>13.8</td>
<td>23</td>
</tr>
<tr>
<td>Moderate-Extensive Fibrosis/EFE</td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td>Median</td>
<td>q1</td>
<td>q3</td>
</tr>
<tr>
<td>Fetal/Pediatric Samples</td>
<td>31</td>
<td>27.1</td>
<td>± 10.7</td>
<td>25</td>
<td>18.5</td>
<td>32</td>
</tr>
<tr>
<td>Adult Samples</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ANOVA analysis was performed comparing LV remodeling association with none-mild fibrosis/EFE to moderate-extensive fibrosis/EFE. Results show an f-ratio value of 11.9 and p-value of <0.001, indicating the results are significant.
Figure 50: Comparison of LV remodeling in relation to the degree of fibrosis/EFE in fetal/pediatric and adult samples.

Fetal/pediatric samples with moderate-extensive EFE had a 10% increase in the mean of LV thickness/length as compared to fetal/pediatric samples that had none-mild fibrosis/EFE. Due to limited prevalence of adult samples with moderate to extensive fibrosis, I was unable to compare LV remodeling in those cases.
Figure 51: Example of an adult heart specimen within the archive included in this thesis. This specimen showed fusion between the right (RL) and the non-coronary (NCL) leaflets. There is calcification present between the non-coronary and the left (LL) leaflets, which does seem to cause fused leaflets. However, no raphe was visible. The blue line shows the margin between the left atrium and the left ventricle, the red line extending from the middle of the mitral valve to the apex was measured as the length of the LV, and the green line placed at 1/3rd distance from the apex of the heart was noted as the thickness of the LV. The red arrows point towards the opaque areas representative of developing endocardial fibroelastosis.
Figure 52: Loss of height of interleaflet triangles.

This is a close up of the same adult heart specimen shown in the preceding figure. Although there is calcification present between both, the right (RL) and the non-coronary (NCL) leaflets and the NCL and left leaflets (LL), the true fusion seen due to presence of a raphe found between the RL and NCL. As a result, note the loss of height of interleaflet triangles (red block arrow).
Figure 53: Calcification seen in adult cardiac sample.

This is another example of an adult heart specimen that was included within this thesis. There is severe calcification present in all three leaflets. Calcification in both the left and the right leaflets obstruct the opening of both coronary arteries which can lead to myocardial ischemia.
Figure 54: Presence of mild endocardial fibroelastosis in an adult sample.

The figure shows an adult heart sample suffering from aortic stenosis as a result of fusion between the right and the non-coronary leaflets. The arrows point towards the opaque areas that are indicative of developing EFE within the left ventricular myocardium.
Figure 55: Valvar raphe in an adult specimen suffering from aortic stenosis

This figure shows a close up of the aortic valve in one of the adult aortic stenosis specimens included in this thesis. The top arrow shows valvar raphe present in an adult aortic valve specimen. The bottom arrow highlights area of opaque pinkish discoloration which was indicative of developing EFE in the LV myocardium.
Figure 56: Pediatric heart specimen suffering from aortic stenosis.

This is an example of one of the cardiac specimens belonging to the pediatric age group that was included in this thesis. The red arrow indicated presence of a (fibrous) raphe present between the right and the non-coronary leaflets. The blue arrows indicate the areas of opacity that were indicative of moderate EFE. Note the loss of the normal myocardial structure within the left ventricle.
Figure 57: Loss of interleaflet triangle in an aortic stenosis specimen

A: The red arrow points towards the fibro valvar raphe present between the right and the non-coronary leaflets. Figure B shows the reduction in the area of the interleaflet triangle as a result of that.
Figure 58: Nodular dysplasia seen in valvar leaflets of pediatric specimens.

The figure shows a close up of the aortic valve of one of the pediatric specimens suffering from aortic stenosis studied within this thesis. This is a unileaflet valve with fusion between the left and the right coronary leaflets, and the right and the non-coronary leaflets. The leaflets shown here exhibit nodular dysplasia often seen in aortic stenosis specimens belonging to the fetal and the pediatric age group.
Figure 59: Left ventricular hypertrophy seen in a neonatal specimen.

This figure shows presence of left ventricular hypertrophy seen in a neonatal specimen suffering from aortic stenosis. The double-sided arrows indicate the measurements of the LV thickness and length taken in all specimens included within this thesis, including this one, giving a ratio index of LV hypertrophy. The blue arrow indicates areas of opacity and whitish discoloration which are representative of moderate-extensive EFE. Note the smoothening of left ventricular trabeculations and the loss of myocardial structure. This is indicative of extensive LV remodeling and severity of disease. Furthermore, the left ventricle is grossly thickened indicating LV hypertrophy. The specimen is a good example of left ventricular remodeling seen in conjunction with severe aortic stenosis.
Figure 60: Fibrous raphe in fetal samples suffering from aortic stenosis.

This figure shows the close ups of the aortic valves of two of the fetal specimens suffering from aortic stenosis that were included as part of this thesis. The red arrows indicate towards the fibrous raphe that are seen at the sites of fusion between leaflets.
3.2.2 Novel Imaging Techniques

11 micro-CT scans of humans, lamb and rabbit hearts were carried out and analysed as part of this project. Similar to measurements taken on gross examination, the thickness, fusion and nature of leaflets, area of interleaflet triangles (ILT), circumference of the aorta, and LV thickness and length were all viewed in these specimens. In addition, use of micro-CT enabled the observation of additional measurements such as, line of attachment of leaflets, sinutubular junction, endocardial thickness, aortic wall thickness and the calculation of the actual cross-sectional area (CSA) of the aorta.

The line of attachment of the leaflets were visible in both Long Axis (LA) and Short Axis (SA) views in 8 out of 11 scans. In 2 scans, however, they were only visible in the SA view and in 1 scan, only visible in the LA view. Sinuses, sinutubular (ST) junction and ventriculo-aortic (VA) junction were seen in all, except 2, of 11 scans (Figure 61).

![Pie charts showing visibility of anatomical junctions in micro-CT scans.](image)

**Figure 61: Visualisation of anatomical junctions on micro-CT scans of the heart samples.**
The line of attachment was visible in both the long axis (LA) and in short axis (SA) in 8 scans (VLA VSA), with 2 only visible in SA (VSA), and 1 only visible in LA (VLA). The Sinuses/ST junction and the VA junction were visible in 9 of 11 scans.

3.2.2.1 Gross versus Micro-CT Measurements

Measurements on micro-CT scans of 4 human heart samples (1 fetal normal, 2 fetal affected, 1 adult affected (Figures 67-70)) were compared to the measurements obtained on gross examination (Figure 62). A ratio of micro-CT:gross measurements was calculated. Micro-CT measurements of fetal samples were less than the values obtained on gross examination. However, the LV thickness:length ratio was multiple folds higher on micro-CT measurements for both abnormal fetal samples, in comparison to gross examination. Conversely, micro-CT measurements of the adult sample was multiple fold higher than Gross measurements, except the LV thickness:length, where the values obtained were the same in both techniques of measurements.

Whilst analysing micro-CT scans, two measurements, the ILT area and the CSA of aorta, were calculated using two different methods. The ILT area were measured by a) using a straight line between the attachment of leaflets as the base, and b) using a curved line that traced the curvature of the aorta. In majority of the cases, the ILT area calculated using a curved based line was more than the area calculated by using the straight line as a base. Similarly, the actual CSA of the aorta measured using the FIJI software using was larger than the CSA of the aorta calculated using the circumference and Pi (Figure 63).
Table 3-12: Statistical Analyses of differences in techniques of measurements of ILT and CSA

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>q1</th>
<th>q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILT Area (curved: straight)</td>
<td>1.5</td>
<td>± 0.4</td>
<td>1.6</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>CSA Aorta (actual: Pi)</td>
<td>1.2</td>
<td>± 0.3</td>
<td>1.1</td>
<td>1.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

ANOVA analysis of ILT area measurements showed $f$-ratio value = 4.7 and a $p$-value < 0.05 indicating that the differences in both techniques were significant.

ANOVA analysis of CSA aorta measurements showed $f$-ratio value = 0.06 and a $p$-value > 0.05 indicating that the differences in both techniques were not significant.
Figure 62: Measurement ratios seen between micro-CT and gross examination for heart samples.

The figure is a graphical representation of the measurement ratio between micro-CT scan and gross examination of 4 human heart samples (1 fetal normal, 2 fetal affected, 1 adult affected). The red line indicates a ratio of 1 between micro-CT and gross measurements. A ratio closer or equal to 1 indicated that there was less or no difference in measurements obtained. Micro-CT measurements of average leaflet thickness, interleaflet area, and cross-sectional area of the aorta of fetal samples were less than the values obtained on gross examination. However, the LV thickness: length ratio was multiple fold higher on micro-CT measurements for both abnormal fetal samples, in comparison to gross examination. Conversely, micro-CT measurements of the adult sample was multiple fold higher than gross measurements, except the LV thickness: length, where the values obtained were the same in both techniques of measurements.
Figure 63: Comparison of the differences in aortic measurements using two different methods.

Differences in measurements was seen when measuring the ILT area using a curved base line versus a straight based line, and when measuring the actual cross-sectional area (CSA) of the aorta versus the cross-sectional area of the aorta using Pi.
3.2.2.2 Differences in Measurements based on the Duration of Iodination

In order to improve the preservation of heart samples micro-CT measurements were taken at various time points during the iodination process; 3, 21 and 28 days. The average interleaflet area and the cross-sectional area of the aorta of samples showed that they decreased in relation to the time they were iodinated. The average leaflet thickness remained the same till 21 days, however, the average leaflet thickness appeared to increase after a further week in Iodine (28 days). Similarly, the average LV thickness and length appeared to increase between 3 days and 21 days in iodine and plateaued afterwards (Figure 64).

ANOVA analysis showed that that $p$-value for all measurements > 0.05, rendering the differences statistically insignificant. However, qualitatively, the images with under/over iodination appeared to be less defined and blurrier.
Figure 64: Comparison of the differences in aortic and left ventricle measurements depending upon the time of iodination.

The measurements of the average interleaflet area and the cross-sectional area of the aorta reduced with longer time in iodine. The measurements of the average leaflet thickness on the other hand remained constant from 3-21 days, however, in the last week, increase by 0.1 mm. The LV length: thickness ratio, increased by almost 10% from 3-21 days in iodine but then plateaued off. ANOVA analysis showed that these results were not significant.
3.2.2.3 Differences in Measurements in Siliconised Samples

A comparison of the differences in measurements of, thickness of leaflets; areas of interleaflet triangles; and, cross-sectional area of the aorta between the control lamb sample and the siliconised lamb sample showed that the mean and median values for the siliconised sample were higher than the control sample (Figure 6.5). Furthermore, the average cross-sectional area of the aorta was greater in the siliconised sample (Figure 6.6).

The mean, SD, and median of the differences in measurements are shown in the tables below (Tables 3.13 and 3.14). One-way ANOVA analysis was performed to compare whether siliconisation of the samples had a significant effect on the measurements obtained.

Table 3.13: Statistical analyses of differences in measurements of leaflets’ thickness between control versus siliconised sample.

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>q1</th>
<th>q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.47</td>
<td>± 0.12</td>
<td>0.45</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Siliconised</td>
<td>0.58</td>
<td>± 0.07</td>
<td>0.6</td>
<td>-</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Table 3.14: Statistical analyses of differences in measurements of area of ILTs between control versus siliconised sample.

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>q1</th>
<th>q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>± 15</td>
<td>23</td>
<td>17</td>
<td>34</td>
</tr>
<tr>
<td>Siliconised</td>
<td>32</td>
<td>± 8</td>
<td>32</td>
<td>24</td>
<td>38</td>
</tr>
</tbody>
</table>
ANOVA analysis of leaflet thickness and ILT area between control versus siliconised samples showed $f$-ratio value = 3.9 and 0.25, respectively, and both had a $p$-value $> 0.05$ indicating that the differences in both techniques were not significant.

However, ANOVA analysis of CSA aorta measurements between control versus siliconised samples showed $f$-ratio value = 7.2 and a $p$-value $< 0.05$ indicating that the differences in both techniques were significant.
Figure 65: Comparison of measurements in control versus siliconised samples.

The measurements of leaflet thickness and interleaflet showed that the mean and median for both parameters were increased by siliconisation. However, ANOVA analysis showed these results to be not significant.
Figure 66: Comparison of cross-sectional area (CSA) of the aorta measurement in control versus siliconised lamb sample.

The average CSA of the aorta measurement was increased in siliconised sample. This difference seen between both samples was statistically significant.
Figure 67: Micro-CT scan of an adult affected heart sample.

The green lines represent the traces of the interleaflet triangles. Whereas, the red lines are the curved base line traced between the pints of attachment of the leaflets along the wall of the aorta.
Figure 68: Micro-CT scan of an adult affected heart sample.

The figure shows the scan as observed in 4 different planes (A-axial, B-coronal, C-sagittal, D-3D) observed whilst using the VGMax Software. The dimensions of the interleaflet triangles (shown in green) were traced, and the area of each triangle was calculated. In section D, note the arrow pointing towards the interleaflet triangle between the right leaflet and non-coronary leaflets which is reduced in height.
Figure 69: Micro-CT scan of a fetal control specimen.

The figure shows the scan as observed in 4 different planes (A-axial, B-coronal, C-sagittal, D-3D) observed whilst using the VGMax Software. The dimensions of the interleaflet triangles (shown in green) were traced, and the area of each triangle was calculated.
Figure 70: Micro-CT scan of a fetal specimen affected with aortic stenosis.

The figure shows the scan as observed in 4 different planes (A-axial, B-coronal, C-sagittal, D-3D) observed whilst using the VGMax Software. The dimensions of the interleaflet triangles (shown in green) were traced, and the area of each triangle was calculated. In section D, note the blue arrow pointing towards the inter-leaflet triangle between the non-coronary (NCL) and left leaflets (LL). The red arrow points towards the right leaflet (RL) and the NCL which is reduced in area. The triangle between the LL and the RL is not shown, as it was not visible to trace. Note the distortion of anatomy as compared to the scan of the fetal control specimen showed in Figure 67.
Figure 71: Micro-CT scan of a control lamb sample

The figure shows the scan of a control lamb sample that has been in Iodine for 21 days as observed in 4 different planes (A-axial, B-coronal, C-sagittal, D-3D) observed whilst using the VGMax Software. The dimensions of the interleaflet triangles (shown in green) were traced, and the area of each triangle was calculated. Note that all three interleaflet triangles (ILT) have been traced, however, the ILT between the left and the right leaflets (red arrow pointing) appears to be a bit distorted.
Figure 72: Micro-CT scan of a siliconised lamb sample.

The figure shows the scan of a siliconised lamb sample that has been in iodine for 21 days as observed in 4 different planes (A-axial, B-coronal, C-sagittal, D-3D) observed whilst using the VGMax Software. The dimensions of the interleaflet triangles (shown in green) were traced, and the area of each triangle was calculated. Note that all three interleaflet triangles (ILT) appear to retain their anatomical shape, and less distortion is seen.
3.3 Development of Novel Bio-prosthetic Heart Valves

3.3.1 DBA staining and Sda expression in GTKO and GMP pig tissues

Pigs show endothelial expression of the B4GALNT2 gene unlike humans and other species which predominantly express B4GALNT2 on epithelial cells. To localize Sda antigen distribution, I used *Dolichos Biflorus Agglutinin* (DBA) lectin to stain tissues from GTKO and GMP pigs. Similar DBA binding was seen in all tissues tested belonging to both GTKO (Figure 73 A-D) and GMP strains (Figure 73 E-H). In the heart, DBA-based staining was present on all vascular endothelial cells throughout the microvasculature within the myocardium of the heart (Figure 73 A, E). DBA binding was variable in lungs depending upon size and type of vessels and was present in endothelial cells lining pulmonary arteries (Figure 73 B, F). In the liver, positive DBA staining was seen on cells lining the liver sinusoids and along the lining of larger blood vessel, hepatic vein (Figure 73 C, G). DBA binding was also seen in the cells lining the glomerular capillaries in the kidney (not shown), along the cells lining the intestinal villi (not shown). A strong positive reactivity was seen to DBA in the spleen due to its increased vasculature and endothelial cells lining the sinusoids (Figure 73 D, H).

3.3.2 B4GALNT2 expression in GTKO and GMP pig tissues

To confirm the presence of Sda antigen suggested by DBA-based immunohistochemical staining, I looked for expression of B4GALNT2 in the pig tissue samples from both GTKO and GMP strains by semi-qualitative RT-PCR. All tissues from both strains expressed B4GALNT2 as evident by the band present at 110 bp. Each tissue also showed a band for ACT-B at 108 bp, which was used as the positive control (Figure 74). Expression was similar in both strains of pigs with spleen
and intestines of both strains showing stronger B4GALNT2 expression compared to heart and other tissues.

Figure 73: DBA Lectin staining of tissues obtained from GTKO (A-D) and GMP (E-H) pigs.

Inserts show negative control of tissues stained with streptavidin only. The image magnification for each section was 20X. A and E: cardiac tissue sections from both species show DBA-based staining present in vascular endothelial cells lining the capillaries within the myocardium. B and F: variable DBA-based staining is seen in pulmonary sections of both species. Staining is present in endothelial cells lining the pulmonary vessels. C and G: hepatic sections from GTKO and GMP show DBA-based staining along the cells lining the liver sinusoids and along the lining of the larger blood vessels, hepatic vein and arteries. D and H: extensive DBA-based staining is seen in both sections of spleen due to increased vasculature. Staining is present in endothelial cells lining the sinusoids and vessels present within the red pulp on the spleen. (Kidney and intestine sections are not shown)
Gel electrophoresis was carried out to test the expression of B4GALNT2 cDNA. Note B4GALNT2 band visible at 110 bp in all tissues in both strains of pigs. The control, beta-actin (ACT-B) band is also visible at 108 bp in both samples. This indicates expression of B4GALNT2 in both GTKO and GMP pigs.

3.3.3 Expression of Sda in PK15 cells

PK15 cells are pig kidney cells that have been used by the McGregor Lab for transfection with recombinant mucin protein, PSGL-1 IgG.
These cells are known to express Gal and Neu5Gc. To determine if PK15 cells express B4GALNT2 and Sda, I used RT-PCR and lectin staining. PK15 cells express B4GALNT2 as evident by the band at 331 bp (Figure 75). Porcine aortic endothelial cells (PAECs), the original source for cloning B4GALNT2 were used as a positive control. These results also show a band at 331 bp.

![Gel electrophoresis of PK15 to measure expression of B4GALNT2 RNA.](image)

Note B4T band visible at 331 bp in both Pig kidney cell line (PK15) and porcine aortic endothelial cells (PAEC). ACT-B band is also visible at 108 bp in both samples. This indicates expression of B4GALNT2 by PK15 cells.

Flow cytometry (or FACS) analysis of PK15 cells stained with DBA was used to confirm presence of Sda antigen. A histogram was plotted with DBA-based staining against the frequency of cells (Figure 76). The negative control cells stained with streptavidin are plotted in blue and the stained PK15 cells binding to DBA are plotted in green. Results indicated DBA binding by PK15 cells and bind to DBA lectin.
Figure 76: Histogram of a FACS analysis of PK15 cells.

This histogram plots DBA-based staining against the frequency of cells. Negative control cells are plotted in blue. Experimental cells that express the antigen are plotted in green. DBA staining is evident which confirms the presence of Sda in PK15 cells.
Chapter 4

Discussion
4.1 Gene Ontology

1 in 50 people born have a bicuspid (bileaflet) aortic valve (BAV), making it the most common form of congenital heart disease (CHD) in the world (Ward, 2000, Evangelista, 2011). Understanding the developmental processes and genetic influences responsible for BAV and aortic valve disease (AVD) in general are crucial in order to improve treatments and increase longevity of these patients (Batsis and Lopez-Jimenez, 2010). Congenital AVD is indicative of underlying genetic factors that may be responsible for altering normal aortic valve development, including over-expression and under-expression of specific genes (Pierpont et al., 2007, Wirrig and Yutzey, 2014).

This thesis has identified and developed a previously under-annotated area of the GO database concerning the proteins involved in development of the aortic valve, and by extension heart valves in general. The annotations documented herein were predominantly assigned to mouse proteins with direct experimental evidence from the literature and transferred to human proteins based on a high degree of orthology.

Based on a thorough review of the literature for genes related to heart valve development, 34 proteins, most of which belonged to either the NOTCH, TGF-beta or WNT signaling pathways, were annotated as part of this project. Data from 27 articles on these genes was reviewed and their attributes captured and uploaded to a bioinformatics database as discussed in section 1.1.4. 353 new annotations, associated with 34 protein entities, were added to the GO database. Out of the 34 proteins studied, 28 were directly annotated to aortic valve development, with the rest of the protein records annotated to other processes involved in heart development. Despite the focus of this project on aortic valve development, more than 100 annotations from the
primary literature have been annotated to other child terms of the much broader parent term “heart development”. This was due to the versatile and widely-observed roles of the signaling pathways in mammalian developmental processes. Whilst, this slowed the annotation process for the specific goal of this project, annotation of all available evidence in the selected literature assists in establishing the mutual pathways within various developmental processes. This not only feeds into the principal goal of the GO database to become a universal repository for functional biological knowledge, but also, allow researchers using GO as a reference tool to come across finding published out of their usual area of expertise, by linking proteins to other areas (Lovering et al., 2018). This allows for identification of proteins that may have not been experimentally proven to be involved in a particular developmental process yet gives researchers new avenues for studies.

A comparison of the Cytoscape analysis carried out prior to and after completion of the project (Figures 64 and 77) shows a large number of the seed proteins annotated to aortic valve development, heart valve development and endocardial cushion development - a crucial process of aortic valve development. Table 3-2 shows 10 GO terms most relevant to this project with aortic valve development being enriched many times after inclusion of annotations created in this thesis. The GO terms associated with these proteins are now a much more accurate reflection of the current state of understanding about the roles of various signaling pathways and proteins involved in the development of the aortic valve.
Figure 77: A comparison of the GO annotations associated with the proteins annotated in this project done prior to and after completion of this project.

The network was created using Cytoscape and the associated GO terms were overlaid using the GOlorize and BinGO apps and 2017 (pre-project) and 2018 (post-project) GO ontology and annotations files. Each node represents a protein, each edge represents an interaction captured in the interaction databases used. The colored segments of the nodes represent the GO terms associated with each protein, see below the networks for a key to the GO term colors. The post-project Cytoscape analysis shows that all proteins annotated as part of this project (highlighted with a red circle) apart from 3 have now been annotated to at least one of the three GO terms representing valvar development. The pink circle around ELN, NOTCH1 and SMAD6 represents the fact that these proteins are included in the Genomics England PanelApp list of genes involved in aortic valve disease; in fact, all 3 proteins in this list have been annotated in this project. The proteins circled in blue represent the protein records not annotated to these three terms as part of this project but were annotated previously in other projects.
4.1.1 Limitations of the GO resource

The GO resource included two dynamic databases that are improved on a day to day basis by the changes and feedback of curators. Although, seemingly a limitation, it does allow for the database to be flexible and adaptable to the continuously changing and ever-developing fields of knowledge and the rectification of previous errors. Therefore, annotation of a previously non-curated area in GO, ‘aortic valve development’, as presented in this thesis, contributes to the growth and refinement, and concepts of GO as a whole.

A more technical limitation, however, encountered during this project was, the inability to annotate the disease processes associated with a particular protein. Since, GO prevents curators from annotating diseases, in cases where genes were evidently preventing a disease process, a different approach to GO was required. For example, in Bosse et al, 2013 and Wang et al 2017, Notch1 is shown to prevent calcification; as there were no terms to annotate the term calcification, nor could new terms be made, the information was, instead, captured using the existing GO term “negative regulation of biomineral tissue development” as calcium is a biomineral, and calcification, a biomineralisation.

Another limitation encountered was during the review of studies of tissue-specific knockouts in mice. Within the existing structure of the GO database, curators are unable to differentiate between annotations based on a full knockout model and those captured from an endocardial-specific, or myocardial-specific knockout. Loss of such additional methodological information during the annotation process is an unfortunate limitation of the project. Although, in order to capture the experimental data in these papers more specifically and accurately, there is provision of an annotation extension
field, which was used in this study, where the site at which this is occurring can be highlighted using the “Cell Ontology (CL)” or tissue ontology (UBERON) terms (Huntley and Lovering, 2017).

One could argue that in essence the goal of GO is to unify the representation of genes and gene products from biological literature, bearing in mind the fact that there is a calculated and planned quid pro quo between the amount of complexity that can be represented versus the ease of user-friendliness and maintainability of the database. GO must be used carefully as a resource tool for research. Researchers must be aware that enrichment analysis to carry out functional profiling of large datasets to assess the proportion of annotations to certain proteins can occur simply by chance; and, as with many fields of bioinformatic study, correction for multiple testing must be properly carried out for meaningful results (Rhee et al. 2008).

4.1.2 Challenges of manual annotation

One of the most fundamental challenges encountered in the process of GO is that of manual annotation. Due to the need of thorough and extensive reading and interpretation of scientific literature, it is very time consuming and the most discernable rate-limiting step in GO (Balakrishnan et al., 2013). A single GO curator can attempt to cover a narrow area of biology involving a limited set of genes over weeks to months, and the database of experimentally-derived results only expands linearly as a function of the man-hours invested by trained and scientifically literate curators. Therefore, a significant proportion of the GO database for the majority of species is built on electronic annotations based on computational algorithms, instead (Huntley et al., 2015). Many of these electronic annotations are based on the transfer of annotations across orthologous or paralogous genes, from model organisms or
human to non-curated genomes. This criticism is somewhat mitigated by the fact that the GO database is increasing over time; meaning, all future work based on GO can benefit permanently from the modest contributions of even time-constrained projects such as this one.

As primary literature is often written with strict word limits, conforming to journal style guides and aiming to convey specific findings over others, the literature, therefore, does not always provide an individual description of the experimental data that it presents, and thus, even with optimal manual curation, GO annotations cannot fully capture the experimental data. This often means that different papers are used to provide a comprehensive analysis of roles of a particular protein. As science is a transforming field, where newer studies allow for the improvement of the existing knowledge, in some cases, previously published data can be outdated. Although, GO now has an automated system for rescinding annotations from a paper that has been formally retracted by a journal, many mistakes made in primary research are not retracted and will continue to be transmitted to the GO database, unless, the new literature is manually curated, and previous annotations either improved or corrected. This of course requires time and manual labor. Furthermore, the current, limited number of experimental evidence codes available in GO do not necessarily reflect the quality or reliability of the research, as different methods and analysis may be more or less reliable. This highlights the fact that since GO curation is one level removed from primary research, GO annotations can only be termed as accurate as the scientific research they are based upon.

This leads to another problem in manual curation, which is that although the literature used for GO annotation is peer-reviewed, GO annotations and GO terms themselves are not formally peer-reviewed. Therefore, even though GO has clear guidelines on
creating annotations and terms, they are enforced primarily through self-policing. This, however, was not an issue in this project as the annotations created were cross checked by senior bio-curators Dr Campbell and Dr Huntley and later reviewed and authorized by Prof Lovering before release to the public database; but, in cases where a curator is working independently, the annotations could inadvertently fall below the expected standards. Consequently, as GO is not a meta-analysis with uniformly applied standard to methodology or analysis, the validity of its annotations is purely based on the judgement of its curators.

The majority of papers studied in this project were those describing mouse models. An obvious question that may arise is why the GO data is going to provide a better interpretation than the mouse phenotype data, seeing as how most of the annotations are based on mouse mutant studies (Eppig et al., 2015). While, in theory this is true, it does not appear to be the case. A search in the MGI resource for the mouse genes associated with the mutant phenotype MP:0000285 ‘abnormal heart valve development’, identified 170 genes associated with this phenotype. However, only 10 of the 18 genes annotated to the GO term ‘heart valve development’ (or child term) as part of the current study were annotated to this phenotype. Of the 24 mouse genes not associated in MGI with the ‘abnormal heart valve development’ phenotype term, at least 8 genes to the GO term ‘heart valve development’ have been created herein. The close evolutionary conservation of mammalian heart development allows for the identification of proteins that are likely to have shared expression patterns between species. Therefore, even in situations where there is incomplete penetrance of a particular phenotype, the data within the GO database can be used for clinical research, for example, the extraction of candidate risk factors for human congenital valvar disease.
4.1.3 Future studies

Based on the information presented in this thesis, there are a number of different directions that future annotations in GO could follow.

Firstly, more work is needed to complete the representation of heart valve development in GO, as illustrated in Figure 78, the convergence of signaling pathways on a few components support the concept of a temporal ordering of these pathways and possible molecular interactions. The annotations created in this project have identified conserved regulatory hierarchies involving signaling pathways, and in particular the proteins belonging to the NOTCH signaling pathways involved in aortic valve development. However, the interconnectivity of the signaling pathways highlights the proteins that have not been curated yet, and that are likely to also play a role in aortic valve development. Using papers that have explored the role of these uncurated yet linked proteins as starting points, the annotation enrichment of the proteins in these pathways would contribute to a more complete representation of functional knowledge of endocardial cushion development in GO.
This network was created using Cytoscape and the associated GO terms were overlaid using the GOlorize and BinGO apps (see methods section). Each node represents a protein, each grey edge represents an interaction captured in the interaction databases used. The red edges (of which there are 6), have been added as part of this project after studying their role in the papers curated. Four of them are from NOTCH1, capturing that NOTCH1 regulates the transcription of SNAI1 and HEY1, HEY2 and HEYL, and two of them are from SMAD4 capturing the regulation of GATA4 and GATA5 by SMAD, within the TGFB signaling pathway. The colored segments of the nodes represent the GO terms associated with each protein: ‘heart valve development’ (blue), ‘aortic valve development’ (red), and ‘endocardial cushion development’ (yellow). The network was created using all proteins that are part of the three main signaling pathways studies in this project, NOTCH, TGFB and WNT. Out of these proteins, the proteins highlighted with a red circle are those annotated as part of this project, and the ones circled in blue represent the protein records not annotated as part of this project but were annotated previously in other projects. The green nodes with no circle indicate that these proteins have not yet been annotated to either of these terms.
Secondly, the annotation of proteins involved in aortic valve development may act as a resource for next generation sequencing (NGS) analysis of patients with BAV (or other valve diseases). By targeted sequencing of these genes, or by using these annotations to filter the identified variants in a patient, it is possible to identify more likely candidate genes contributing to disease. The majority of NGS analysis tools currently use mouse phenotype data, or the Genomics England PanelApp data from the 100,000-genome project (https://panelapp.genomicsengland.co.uk/). However, a simple knockout of these mouse genes may either be embryonic lethal or not affect the valve due to the presence of paralogs or other genes which can override the mutation (or have incomplete penetrance, affecting only a limited number of mice), therefore, it is more appropriate to use human genes for genomic analyses. Three of the 28 proteins annotated to aortic valve development, ELN, NOTCH1, and SMAD6 have been identified by the Genomics England PanelApp, to be involved in aortic valve disease (AVD) (Figure 79). The gene list studied in this thesis could provide substrates for future genomic studies and expand the scope of virtual gene sharing platforms such as the Genomics England PanelApp, thus help clinicians and researchers alike in gaining a better understanding of AVD. In addition, some malformations of heart development i.e. congenital heart diseases have already been identified as due to polygenic causes (Galton and Ferns, 1999, Kathiresan and Srivastava, 2012, Gelb and Chung, 2014). Therefore, variants present within multiple components of a related signaling pathway may increase the likelihood that these are working in a polygenic way to cause the defects.
Figure 79: Genes associated with AVD in Genomics England PanelApp.

Only three of the 55 genes listed in the Genomics England PanelApp involved in familial nonsyndromic congenital heart disease have been associated with aortic valve disease. The green indicates a high level of evidence for this gene-disease association, demonstrating confidence that this gene could be used for genome interpretation. The amber indicates moderate evidence for this gene to disease association and should not yet be used for genome interpretation. The red indicates not enough evidence for this gene to disease association and the gene should not be used for genome interpretation.

A further interesting field for annotation would be the role of the various signaling pathways in adult aortic valve disease. So far, our understanding of aortic valve disease presented in adults is limited based on the etiology; congenital or acquired. The genetic basis of adult aortic valve disease still remains uncharted territory with immunogenicity being blamed as the main culprit for calcification. As seen in this project genes such as NOTCH1, RB1 and TGFB1 not only play a role in the development of aortic valve, but also appear to prevent valve calcification. Furthermore, the Wnt signaling pathway genes, ROCK1 and ROCK2, have also been identified as preventing calcification, however, as of yet, no role has been established of ROCK1 and ROCK2 in aortic valve development. An understanding of the role of
signaling pathways involved in adult AVD could be achieved by comparing the transcriptome or proteome of explanted calcified aortic valves from adult patients undergoing aortic valve replacement surgery, with noncalcified aortic valve transcriptome or proteome. This may lead to the identification of additional genes involved in adult calcific AVD. Targeting additional Wnt, NOTCH and TGFB signaling pathways, using mouse knockout studies, or explanted valve models, followed by ontological analyses of the subsequent transcriptome or proteomes would help provide a biological, functional and cellular framework of these genes. This could help in creating gene targeted therapies that can help combat aortic valve calcification and disease.

4.1.4 Conclusion

In this project, the role of NOTCH, TGF-beta and Wnt signaling pathways along with other proteins, in aortic valve development has been documented in the GO database using 27 sources of primary literature. This has led to the submission of 353 new annotations, associated with 34 unique protein entities, to the GO database. Comprehensive annotation of these biological functions allows this area of scientific understanding to be included in statistical analysis of datasets using the freely available GO database. This is an increasingly important tool in the modern genomic era of high-throughput technology and big data.

The future utility of the GO database depends on updates and refinements such as those carried out in this project to maintain a current and broad-scoped knowledge base. There are several related subject areas, such as pulmonary valve development, mitral valve development, and left ventricular development and hypertrophy, which could be comprehensively annotated to improve the GO database’s relevance to valve disease.
and congenital heart disease as whole. With continued collaboration between bioinformaticians, basic scientists, and clinicians, these incremental improvements in understanding may eventually help develop a better understanding of aortic valve disease and the therapeutic outcomes for patients affected by these diseases.
4.2 Cardiac Morphology

Heart valve dysfunction is a significant cause of cardiovascular disease, with more than a million patients in the UK alone (www.statistics.gov.uk). Aortic Valve Disease (AVD) is the most common cause of heart valve disease in the Western World, affecting an average of about 3-4% of the Western population (Hufnagel et al., 2015, Osnabrugge et al., 2013). Causes of AVD can be congenital, such as BAV or acquired, e.g. age-related degeneration of the aortic valve and functionally, AVD results in narrowing of the aortic valve, aortic stenosis, or, the inability of the valve to close completely, aortic regurgitation. Aortic Stenosis (AS) presenting in the neonatal period due to congenital malformation of the aortic valve contributes to two-thirds of obstructive lesions affecting the left ventricular outflow tract (Carr et al., 2018). In order to gain a better understanding of the disease process across the life-course, from early life to adulthood, 56 heart samples with AS were examined, out of which, 6 were fetal, 37 were pediatric below the age of 6, and the remaining 13 were adult samples ranging in age from 18-80 years.

4.2.1 Valve structure

Three morphological variants of AVD were identified in this study: unileaflet aortic valves (UAV), bileaflet aortic valves (BAV) and tri-leaflet but stenotic aortic valves (TAV). Previous studies have frequently identified ‘bicuspid aortic valve’ as the common congenital aortic valve malformation with heterogenous morphologic phenotypes (Fernández et al., 2009, Sabet et al., 1999, Sievers and Schmidtke, 2007). However, the widely prevalent practice of using the term ‘cusp’ instead of ‘leaflet’ is not only a misnomer but often leads to misperceptions when studying the morphology of AVD. Its use has often led to the gross underrepresentation of specific phenotypes
such as UAV, which in turn leads to a lack of understanding the pathophysiology of AVD, as a whole. A correct understanding and description of the detailed morphological variants of aortic valves and their development is, therefore, necessary in order to tackle the challenges in the treatment of AVD and to determine etiologies.

Studies have shown ‘bicuspid AV’ is one of the most common congenital heart defect affecting about 0.5 - 2% of the population, whereas, studies characterising UAV as an independent entity have found it to be a rare form of aortic valve malformation, with a prevalence of 0.02% in the adult population (Fernández et al., 2009, van Engelen et al., 2014, Novaro et al., 2003b). Amongst the aortic stenosis samples included in the current study, 73% were UAV, 25% were BAV, and only 1 sample (2%) was TAV. Comparison of fetal, pediatric and adult samples found that the ratio of UAV to BAV in fetal and pediatric samples was 6:1, whereas, the ratio in adult samples was 5:7. The incongruity between the prevalence of UAV and BAV found in this study and the statistical data found in published literature could have two possible explanations. First, (as mentioned above) many studies have grouped UAV with BAV, under the umbrella term, ‘bicuspid aortic valve’, therefore bicuspid AV will be statistically more common. Second, the reported mortality is higher in patients with UAV, with fewer patients reaching the age of adulthood, and they are therefore, not accounted for in published statistics of older cohorts (Falk et al., 2017, Singh et al., 2015, Novaro et al., 2003a). Since the current study was based on post-mortem samples, predominantly belonging to the fetal and pediatric age group, and the morphology of the aortic valve was documented in detail, an increased incidence of UAV was found. The failure of patients with UAV to reach adulthood indicates that, not only is the disease severe, but also suggests that it may differ in aetiology. Since UAV patients die early and are often coupled with major systemic complications, studies that have characterised UAV
disease in humans are fairly limited. Recent studies in mice, designed to recapitulate the morphological findings of UAV in human, have demonstrated a progressive increase in aortic valve dysfunction and structural adaptation (Weiss et al., 2018). Interestingly, the UAV in this mouse model were malformed and dysplastic but had no calcification, while those with a BAV, had a normal leaflet structure, albeit, having only two leaflets. These findings mirror my own observations of valve leaflet structure in humans where the majority of UAV samples were dysplastic, again suggesting that UAV may have a different aetiology to BAV. Mechanically, the mouse model described above showed that cumulative tissue responses disturbed flow over time – for reasons yet to be discovered – and showed extensive LV remodeling indicating that LV responses to initiation of hemodynamic stress early in life, as seen in UAV, differ significantly from LV responses due to hemodynamic stress seen later in life (which maybe the case in BAV). Again, this supports the premise that UAV may be a distinct phenotype (from BAV) and strengthens the argument for the need of precise phenotypic descriptions, in order to identify the morphological variants of AVD and, subsequently, link to genotype.

The phenotypes of UAV and BAV are characterised on the basis of the spatial alignment of the commissures between the free leaflets, and the site(s) of fusion between the leaflets that are fused. Three morphologic variants of UAV were identified in this thesis. By far the most common phenotype, found in 83% of the samples, was with fusion between the Left Leaflet (LL) and Right Leaflet (RL), along with fusion of the RL and Non-Coronary Leaflet (NCL). Other phenotypes were present in significantly smaller populations (RL-NCL and NCL-LL - 12%; NCL-LL and LL-RL - 5%). A similar trend was found on analysis of BAV samples in the current study, where 57% had fusion between LL and RL (the remaining 37% had fusion between
RL and NCL, and only 7% showed fusion between NCL and LL). Albeit, a smaller sample size, (with consequently wide confidence intervals), the phenotypic trend seen in these samples mirrors that in previously published morphologic and imaging studies (Bissell et al., 2013, Mahadevia et al., 2014, Sievers and Schmidtke, 2007). During development, the leaflets of the aortic valve, together with their respective sinuses are sculpted from the intermediate segment of spiralling outflow tract cushions which fuse to divide the developing outflow tract, along with an additional aortic intercalated cushion (Anderson et al., 2016). The higher incidence of fusion involving the RL in both UAV and BAV may be due to the fact that the RL originates from the more anterior of these outflow tract cushions, whereas the LL and NCL originate from the more posterior and aortic intercalated cushion respectively (Anderson et al., 2016). Consequently, it may be that there are differences in gene expression or temporal development of each of the three cushions including differences in epithelial to mesenchymal transition (EMT); responses to blood flow; or, shear stress (Fernández et al., 2009) that are the substrate for abnormal development. Alternatively, the relatively high proportion of valves with fusion between the coronary leaflets could indicate abnormal fusion of their precursors (the intermediate part of the anterior and more posterior outflow tract cushions) during outflow tract ‘septation’. Similar findings have been reported in specific animal models, such as the Syrian Hamster (Sans-Coma et al., 1996).

Correlation of the patterns of leaflet fusion to the presence of endocardial fibroelastosis (EFE) showed that the majority of samples had LL-RL fusion; 83% of which were associated with moderate-extensive EFE. Due to the low number of samples (n=7) having no fusion between LL-RL, a significant correlation between the presence of LL-RL fusion and severity of EFE could not be established. A repeat analysis with a
greater number of samples may reveal whether the presence of LL-RL fusion can serve as a diagnostic indicator of endocardial phenotype. Nonetheless, the observation noted in this study questions whether the fusion between LL-RL results in left ventricular remodeling or whether the abnormal fusion is a result of an initial left ventricular abnormality. Alternatively, an abnormal endocardial cell phenotype may be the common link between the two (Xu et al., 2015). Genomic studies on samples with left ventricle remodeling along with LL-RL fusion could help provide a better perspective of the possible link between these entities.

In the normal fully developed aortic valve, each leaflet is attached to the wall of the aorta via a semi-lunar line of attachment (Figure 12). Through this line of attachment and the lunula shape of the leaflets, each leaflet opposes its adjacent counterpart during ventricular diastole, forming three zones of apposition termed commissures which extend from the wall of the aorta and join centrally. The central junction is often evident on each leaflet by the nodule of Arantius or even finger like endocardial projection, termed Lambl's excrescences (Duran et al., 1995, Anderson et al., 2009). When the leaflets are fused in cases of abnormal aortic valve development, there is loss of one or more commissures, which are then replaced by the formation of a ridge or raphe (Sutton III et al., 1995). In the past, studies have classified BAV based on the loss of commissures and grading of the raphae (Sievers and Schmidtke, 2007). However, no thorough studies have been reported explaining the nature of raphae in cases of UAV. The nature and composition of raphe will likely affect the mobility of the leaflets, which, if they do not fall back into the sinuses adequately during systole, will cause stenosis. An in-depth understanding of the nature of the raphae found in abnormal aortic valves, could therefore aid in making pre-operative decisions regarding surgical interventions to relieve stenosis, especially in neonates and
pediatric patients, where repair rather than replacement is paramount. In the current study, a detailed analysis of the composition of raphae with respect to the site of fusion was therefore carried out. The vast majority of the raphae present in fetal and pediatric samples, were fibrous in nature, being composed mainly of an infolding of the aortic wall. A small proportion of raphae showed both fibrous and valvar components, and a miniscule number of raphae consisted entirely of valvar tissue. The latter were predominantly found in raphae existing between NCL-LL (still forming a mere 14% of the fetal and pediatric NCL-LL analysed). In contrast, none of the raphae seen in adult samples were entirely fibrous in nature, with the majority being valvar. The raphe found between NCL and LL in adults were always entirely valvar. Whether the NCL-LL raphe has a slight predisposition to develop from valvar tissue, and if so, whether this reflects differences in gene expression of their precursors (the endocardial cushions) or the mature leaflets; or, whether it reflects the nature of blood flow across these leaflets during later life, is difficult to say. Further studies into developmental mechanisms and gene expression patterns of the individual endocardial cushions and of mature leaflets of the aortic valve with a large sample size will be necessary, in order to provide a better understanding of the characteristics of leaflet fusion, and the formation of raphae. Ideally, such studies should also be linking these findings to characteristics of blood flow. Although studies have previously described the raphe as being a ‘fibrous ridge’ (Angelini et al., 1989, Leung et al., 1991, Sutton III et al., 1995), the study conducted herein provides a greater appreciation of the variability that exists in the composition and nature of raphae themselves. Using the data shown in this work, it would seem appropriate to incorporate a structural classification of raphae in conjunction with the existing method of grading raphae, used by Sievers et al 2007.
Previous studies have also shown that the structure and composition of the valvar leaflets are altered in cases of aortic stenosis (Angelini et al., 1989). In AS samples, leaflets are thickened and nodular which some suggest could be due the abnormal hemodynamics found in these patients, through exposing the leaflet to increased stress and vibration (Maizza et al., 1993). 65% of the fetal and pediatric samples studied did not have calcification or nodularity, whereas the remaining 35% had nodularity of various degrees present on their leaflets. In adult samples, 46% exhibited calcification ranging from mild-severe, 8% had nodularity, and the remaining 46% had no calcification or nodularity. This indicates that the nature of the expression of disease, and therefore the aetiology, varies in fetal and pediatric versus adult specimens, as calcification which hugely impacts stenosis in adults, was not evident in any of the fetal and pediatric samples either grossly or by high resolution X-Ray micro-CT imaging.

4.2.2 Interleaflet triangles

The final key structure of the valvar complex, the anatomical understanding of which is crucial for improving the surgical approach in reconstructing congenitally malformed valves and valve replacement, are the interleaflet triangles. Since the valvar leaflets are normally attached within the aortic root in a semilunar rather than annular fashion, there are thin layers of tissue underneath the apex of each commissure that exist between each of the leaflets which have been termed interleaflet triangles (ILTs) (Anderson et al., 1991). In the normal aortic valve, these spaces extend from the tops of the aortic sinuses to the basal attachment of the leaflets within the left ventricle. The ILTs are crucial for proper valvar development and function of the aortic valvar complex, and have been noted to be lost or reduced in malformed valves (Sutton III et al., 1995, Tretter et al., 2016). Some surgeons have even suggested that ILTs are
primarily responsible for the development of AS, especially in neonates and children, as, during surgery, they note the ILTs are severely malformed and distorted (George Belitsis – Personal Communication). Therefore, when studying AS, it is important to conduct a full assessment of the ILTs in order to improve our understanding of the functional capability of the entire aortic valvar complex. Although, the ILTs do not possess the shape of a perfect arithmetic triangle, due to the semilunar attachment of the leaflets and curve of the adjacent aortic sinuses, for purposes of this study, the area of each ILT was calculated assuming it approximated a triangle. Previously, some authors describe the base of each interleaved triangle as being formed by the anatomic ventriculoaortic junction, and the sides by the hemodynamic ventriculoaortic junction marked by the hingepoints of adjacent leaflets (Angelini et al., 1989, McKay et al., 1992, Sutton III et al., 1995). Others extend the boundaries more proximally to the base of the aortic root (Tretter et al., 2016). In the current study I chose to maximize the boundary of the interleaved triangles in the manner of Tretter et al. and define the base as the distance between the nadir of each adjacent sinus (Figure 20). I reasoned that, in reality, the leaflets originate well below the anatomic ventriculo-aortic junction, at what some authors have called a ‘virtual basal ring’ which expands in its entirety during systole. Although the tissue beneath the anatomic VA junction may be subject to left ventricular pressure, studies have shown that the expansion of the aortic root is not restricted to the component above the anatomic VA junction, but rather extends from the base of each of the sinuses (Tretter et al., 2016). I, therefore, hypothesised that it would be incorrect not to include the area underneath anatomic VA junction when calculating the ILT since this area contributes significantly to the function of the aortic valvar complex. Furthermore, when using the anatomic VA
junction as a base of the ILTs, the size of the ILT will be reduced significantly making comparison with the small ILTs seen in stenosed valves more difficult.

In the current study the height of the interleaflet triangle was measured from the base of the interleaflet triangle up to the highest point of each commissure. In malformed valves where the commissure was lost and replaced by a raphe, this height was consequently reduced with the point of fusion of both leaflets noted as the highest point of each triangle. I then chose to create a ratio of the area of each ILT to the cross-sectional area of the aorta (CSAA) for standardization of the measurements across different age groups and heart size. Comparison of this calculated ratio in relation to the presence and type of raphe showed significant differences. In fetal and pediatric samples, the mean of the ratio (ILT:CSAA) in unfused leaflets was 0.36 as compared to an average of 0.05 in cases where a raphe was present. Similarly, in adult samples, the mean ratio in unfused leaflets was 0.3, 6 times greater than the average mean found in cases where raphe was present. Furthermore, in fetal and pediatric samples, those cases with a raphe consisting of valvar components, had an average mean of 0.05, more than twice the mean found in the setting of purely fibrous raphe. Since none of the adult samples demonstrated the presence of fibrous raphe, I was unable to test whether a similar trend was seen in these samples. Although, previous studies (Sutton III et al., 1995, Tretter et al., 2016) have described that the presence of fusion of leaflets significantly reduces the area of the ILT, contributing to valve dysfunction, this study is the first of its kind to correlate the composition/nature of the raphe to the area of ILTs. This correlation may aid clinical assessment by ultrasound of the presence of valvar tissue within raphae through assessment of the size of the ILTs, thereby aiding the surgeon in valvar repair in patients with AS.
4.2.3 Left Ventricle

As narrowing of the aortic valve progresses, preventing it from opening fully, the left ventricle adapts to the increased resistance first through dilatation; then hypertrophy and eventually, fibrosis, which are important determinants of prognosis in patients with AS and potential indicator of irreversible LV dysfunction (Azevedo et al., 2010). To evaluate the remodeling response of the left ventricle in this study, in relation to the phenotype of the aortic valve, I chose to measure an estimate of the mass of the left ventricle. During life, LV mass is typically assessed in patients with aortic valvar stenosis using echocardiographic parameters in comparison to their body mass. There have been no recent (gross) morphological studies that have looked in detail at the left ventricle in aortic valve stenosis, so I chose the parameters closest equivalent to current echocardiographic measurements in terms of morphological assessment: the thickness and length of the LV wall (Devereux et al., 1997, Pouleur et al., 2008). A percentage ‘index’ was calculated to standardize the measurements and account for the variability seen amongst samples of different age groups. Once the LV mass was assessed and presence of fibrosis noted, two key observations were made; first, moderate to extensive fibrosis was more prevalent in the fetal and pediatric age group with a collective incidence of 74%, almost twice as high as the 40% seen in adults; secondly, the degree of fibrosis correlated to the presence of number of functional leaflets. UAV showed a higher predisposition to fibrosis than BAV, with 73% of UAV samples showing moderate to extensive fibrosis. The reverse being true in BAV samples, where 73% had none to mild fibrosis. Since fibrosis is considered the endpoint of LV remodeling and, therefore, related to left ventricular hypertrophy (LVH), I performed, for the first time to my knowledge, a morphological assessment of LVH in relation to fibrosis in aortic valve stenosis. A comparison of the fetal and pediatric age samples...
showed that the mean LV index seen in samples that had none or only mild fibrosis was 16.7%, as compared to a mean LV index of 27.1% in samples with moderate to extensive fibrosis, almost 1.6 times higher. This suggests that LVH correlates with a greater degree of fibrosis with the latter being the endpoint in LV adaptation. Conversely, we still cannot exclude the possibility that a more severely affected and dysfunctional LV could be the precursor to more severe aortic valve disease during early development (Sharland et al., 1991). I was also not able to assess, using current techniques, whether other LV abnormalities, such as amyloid, are a component of LV disease. These are seen as increasingly important in adult patients with aortic valvar stenosis (Scully et al., 2018). Further techniques using high resolution imaging as explored in this thesis, along with the use of appropriate biomarkers, may well be able to detect these components in the future.

4.2.4 Novel Imaging techniques

Micro-focus CT (micro-CT) is a promising, and novel, state-of-the-art imaging tool for phenotyping and interpretation of disease and by multiscale analysis helps to bridge the gap between microscopy and gross examination (Hutchinson et al., 2016, Hutchinson et al., 2017). The high resolution imaging of specimens carried out as part of this thesis is the first of its kind to demonstrate the feasibility of analysing the anatomy of the aortic valvar complex in human hearts in 3D, providing a comparison between gross versus micro-CT measurements, and provides a baseline for further development of the technique. Protocols were optimized first using lab and rabbit hearts, serving as a model for adult hearts and fetal human hearts, respectively, due to the similarity in anatomic size and structure. 8 out of 11 (73%) scans were of high quality and allowed clear visualisation of key specific anatomic features of the aortic root and LV (as described in results section). However, two of the heart specimens (1
rabbit, and 1 abnormal fetal heart) produced sub-optimal imaging. Of many possible factors the most likely reasons are poor penetration of iodine or sub-optimal tissue morphology resulting in loss of image quality. Further work, to determine the factors involved are warranted but due to time constraints were beyond the current scope of this thesis.

4.2.4.1 Gross versus micro-CT measurements

4 of the human heart specimens (1 normal fetal, 2 abnormal fetal, 1 abnormal adult) were selected in order to compare measurements of the aortic root taken by gross and micro-CT examination. The results show that there were indeed observed differences between the two techniques in multiple indices. Most measurements obtained by micro-CT were less than those acquired by gross examination. This may be due to the greater resolution provided by X-ray imaging, especially for smaller sized fetal hearts, as compared to measurements taken with, for example, Vernier callipers on the gross sample. However, some shrinkage undoubtedly also occurs due to process of iodination (Vickerton et al., 2013).

One characteristic, the LV thickness: length index - which is a ratio coined within this study to serve as an indicator of LV remodeling - showed micro-CT measurements of LV lengths in both fetal abnormal samples that were less than those observed on gross examination. This likely highlights that it had not been possible to appreciate the dimensions of the LV on gross examination with the same degree of precision as micro-CT due to the small size of the specimens. An accurate assessment of LV remodeling is clinically significant as greater remodeling (i.e. hypertrophy and EFE), is an indicator of increased disease severity. It is interesting to note that the normal fetal sample showed no difference between micro-CT and gross measurements in
terms of LV thickness: length, possibly due to absence of LV remodeling. A similar observation was made in the adult sample, which despite having a bi-leaflet aortic valve, did not have moderate-extensive LV remodeling and, therefore, showed no difference between both techniques. Whether this has any statistical significance is hard to say and further studies using a wider variety of samples to measure the effect of iodination in fibrotic and non-fibrotic tissues are needed to resolve this question.

Overall, the comparison between micro-CT and gross measurements indicated that there are differences between measurements which should be taken into account by morphologists, surgeons and radiologists, in future studies, as there might be significant differences in the anatomic dimensions of the aortic valvar complex between the pre-operative imaging studies and findings observed anatomically, or during surgery.

4.2.4.2 Optimization of micro-CT imaging

The 3D nature the micro-CT datasets also allows comparison of different types of measurement of the aortic root to be performed and emphasises the need, where possible, to move away from iodine contrast enhanced imaging.

In this study, for example, the area of the ILT and the CSA of the aorta were calculated using two different methodologies using either the arc of the aortic sinuses/root or the cord measured across the same arc (as described in methods section) The differences seen in ILT area measurements between the techniques were found to be statistically significant with a $p$-value of $<0.05$. The technique measuring the arc most closely matched the method carried out on gross examination, and the ILT areas calculated using this method were found to be greater than the method using the cord. This suggests that meticulous attention needs to be paid by surgeons and cardiac imaging
experts when measuring the area of the ILT preoperatively using imaging techniques, if results are to be consistent and aid in making surgical decisions about the size of the whole aortic root. Due to the ability of the imaging software to measure the (cross sectional area) CSA, I was also able to obtain two values for the CSA of the aorta in micro-CT datasets, the actual one, and the CSA derived using the circumference of the aorta. The latter technique was based on the method for measuring the CSA of the aorta on gross examination. Since the difference between both techniques was statistically insignificant, this provided verification of the validity of the measurements I had calculated on gross examination.

In order to optimise the approach used for novel imaging techniques of cardiac specimens, I looked into the effect of prolonged iodination and the possibility of using silicon as a method for preserving the shape of the specimen, which is known to distort after prolonged iodine exposure (Vickerton et al., 2013). Imaging was carried out after 3, 21, and 28 days of iodination and while changes were observed in measurements at different time points, they were not statistically significant, with the caveat that the number of scans was low. A larger scale study may be able to assess further the effect of iodination on cardiac archive specimens. Nonetheless, qualitative analysis showed that scans carried out at 21 days in adult hearts were optimal in terms of image quality, contrast and definition. Scans carried out at 3 and 28 days of iodination had reduced- and over- exposure, respectively, making it difficult to visualise the cardiac anatomy, demonstrating the possibility of the phenomenon of both, under- and over-iodination.

I also explored the effect of siliconisation as a method to preserve the shape of the aortic root as the use of such methods in archival cardiac specimens has not been explored in previous studies and there had been some suggestions from our research group that distortion could occur to finer structures. Although siliconisation did not
significantly alter the area of the ILT and the leaflet thickness, it provided preservation of 3D anatomy of the aortic root and in consequence made the measurements on micro-CT datasets easier to carry out. Furthermore, the CSA of the aorta was (statistically) significantly larger in the siliconised sample demonstrating the ability of the silicon to counteract shrinkage of the cardiac tissue due to iodination. Overall, the findings of the micro-CT study indicate the need to move away from iodine contrast imaging, and, in the future, phase contrast micro-CT imaging (without contrast) may well provide the means for high resolution imaging with minimal distortion (Zamir et al., 2016).

4.2.5 Implications on surgical intervention

The detailed structural classification of raphe and relationship to ILTs described in this thesis provides surgeons with an insight into the likely nature of raphe prior to surgery. By determining the likely nature of raphe and the area of the corresponding ILTs and taking into consideration the age of the patient and site of fusion, it may be possible to assist them in making informed decisions regarding valvular repair or replacement. Surgically, the thickness and structure of raphe would determine the quality of leaflets that can be utilized in repair; better-quality native tissue increases the chances for a successful aortic valve repair, which includes various techniques such as commissurotomy, annuloplasty, debulking and free edge unrolling and ‘neocuspidization’ (Ozaki et al., 2018, Kalfa et al., 2019). Conversely, the poorer the quality of leaflet tissue and less valvar the raphae, the less suitable the leaflet would be to act as a substrate for aortic valve reconstructions, therefore, making valvular repair in such cases more challenging and less likely (Takatoh et al., 2011). A pre-surgical assessment of the nature of ILT tissue could aid in making informed decisions when considering aortic valve repair using these procedures.
4.2.6 Limitations

4.2.6.1 Gross Morphology

Some of the data presented in this section have a broad range of confidence intervals due to a relatively small sample size. This is one of the limitations of the study, but the trends shown provide good rationale for expanding the research and collecting data from specimens with aortic valvar stenosis held in other archives in the UK and even worldwide.

20 specimens were selected for repeat measurements to check for inter- and intra-observer variability of two measurements: LV thickness and LV length. Although there wasn’t high variability between the observations, ideally, all measurements and observations noted should have been checked for inter- and intra-observer variability.

Studies show that formalin fixation may cause shrinkage of specimens (Tran et al., 2015). As the specimens were all post-mortem, and some in fixative for decades, I was unable to take into account of the effect of formalin on tissue over time and the assumption in this was that any effects (for example shrinkage) would have reached a steady state. Future studies could be performed to assess the degree of shrinkage starting with fresh tissue and the effect on measurement either grossly or through micro-CT.

Ideally, each AS heart sample examined should be compared to a normal control matched in sex, age, size, and time of fixation, amongst other factors, however, such material was not available for study. I circumvented this issue by using each heart sample as its own control in measurements, and calculated ratios rather than absolute values, which helped standardize the measurements that showed wide variances due to age.
4.2.6.2 Micro-CT

One of the most important limitations of micro-CT imaging was the low number and narrow age-range of the specimens. The low number may have altered the statistical significance of variations seen in measurements of leaflet thickness, interleaflet area and LV thickness:length ratio, amongst others. Furthermore, the human cardiac specimens studied were from the historic collection at the ICS Cardiac Archive and have been dissected for morphologic examination and clinical correlation. Due to this, it was often difficult, and in 1 of 4 cases not possible, to study some of the intricacies of valvar and ventricular anatomy. Given the distortion this can produce, as demonstrated in this study, it would be better to perform micro-CT studies on intact specimens prior to dissection wherever possible.

Furthermore, no inter- or intra-observer variability were performed for micro-CT measurements, there is potential for human error. However, errors were minimized through appropriate guidance and verification of the methods by Prof Cook and Mr Simcock.

4.2.7 Conclusion and Future Work

This study is the first, to my knowledge, that combines gross examination of aortic valvar stenosis with high resolution imaging providing important insights into the progression of AVD. Future studies using novel imaging techniques to compare with finding on gross examination could help in giving further insights of the morphology of the abnormal aortic valve.
4.3 **Xenogeneic Heart Valves**

Aortic valve replacement (AVR) is the second most frequent cardiac surgical procedure after coronary artery bypass graft (CABG) surgery (Clark et al., 2012, Bonow, 2013). The growing numbers of patients undergoing this procedure are primarily due to the development transcatheter-based approaches to AVR which allows for AVR to be carried out in octogenarians and nonagenarians who are otherwise considered high-risk candidates for surgical AVR (Leon et al., 2010, Kodali et al., 2012). Valvar replacement options include, surgical valves consisting of a frame with mechanical or biological valve leaflets; surgical reconstruction of valve leaflets using homograft, xenograft or non-biological tissue; and trans-catheter aortic valves comprising a xenograft heart valve sutured to a balloon expandable or self-expanding metal frame.

Biological tissue valves are preferred over mechanical valves, and their use is currently increasing in frequency, due to the generally low level of thrombogenicity. With the rising trend of trans-catheter procedures which uses biological tissue valves, and the number of surgeries in low risk older patients, the need for durable biological heart valves is ever more important.

One disadvantage of xenogeneic biological heart valves (BHV) is structural valve degeneration (SVD) which is accelerated in younger patients (Manji et al., 2012a) and is the most common cause of reoperation in such patients. The major pathology of SVD is tissue calcification which promotes leaflet tearing and valve obstruction (Zilla et al., 2008). There are multiple factors which contribute to tissue calcification, but calcification induced by antibody reacting to xenogeneic glycans enhances age dependent BHV degeneration. This occurs because xenogeneic glycans on these bio-
prosthetic xenograft valves are not masked by glutaraldehyde fixation (Byrne et al., 2015).

Studies have shown that xenogeneic glycans are present on current commercial bio-prosthetic heart valves of both bovine and porcine origin and remain immunogenic (Konakci et al., 2005, McGregor et al., 2011, Park et al., 2012). They further show that human antibody binding to Gal, the major xenogeneic glycan enhances tissue calcification, suggesting that use of Gal positive bio-prosthetic valve tissue during valve replacement or repair can induce an anti-Gal antibody response and, thereby, increase the likelihood of BHV degeneration (Byrne et al., 2015). This illustrates the potential for other human antibodies binding to xenogeneic glycans to further affect BHV durability. However, even less is known about antibody reactivity to other glycans, such as Neu5Gc and Sda. This is because Neu5Gc and Sda have been unappreciated immunogenic agents until recently (Byrne et al., 2018). Furthermore, immunogenicity against these non-Gal glycans is unknown because of poorly developed assays that do not measure the immunogenicity of all xenoglycans in a uniform manner (Byrne et al., 2018).

4.3.1 Expression of Sda in porcine tissues

Sda, a minor blood group antigen, has been demonstrated to be xenogeneic in pig to non-human primates (NHP) cardiac xenotransplantation. Sda is made from the galactosyltransferase, B4GALNT2 which in pigs is expressed on endothelial cells. In this thesis I have shown a strong correlation between porcine tissues stained through DBA-based immunohistochemical staining – due to its specificity toward alpha-linked N-acetylgalactosamine – and B4GLANT2 gene expression indicating that the Sda antigen is indeed expressed in bio-prosthetic tissues including pig pericardium.
Expression of B4GALNT2 in pigs is somewhat unusual as in humans, and most murine, bovine, and ovine strains; B4GALNT2 is predominantly expressed in epithelial cells in the gastrointestinal tract and the kidneys, with B4GALNT2 expression notably absent from endothelial cells. I have shown that DBA-based staining and B4GALNT2 expression in a laboratory inbred pig strain, Gottingen mini-pig (GMP) is similar to the Gal-knockout (GTKO) pig. The GTKO pig is a common agricultural pig strain (White Landrace). DBA-based immunohistochemical staining and B4GALNT expression was evident in this strain. These results agree with previous DBA studies showing endothelial cell binding of DBA in commercially available pigs, Yucatan mini pigs, and Pan-pinto micro pigs (Darr et al., 1990, Johnson et al., 2002) and suggests that endothelial expression of B4GALNT2 in pigs shows no or very limited polymorphism in B4GALNT2 expression in pigs.

4.3.2 Conclusion and Future Work

Since B4GALNT2 is present in all strains of pigs, and therefore, all porcine tissues have xenogeneic glycans present on their surfaces, it is essential to have a standard uniform assay to measure antibody reactivity to all three xenogeneic glycans. This assay is needed to compare the prevalence of these antibodies in human serum and to monitor changes to antibody levels to these glycans in patients after BHV replacement surgery. This would confirm whether Sda and Neu5Gc are immunogenic and thereby, along with anti-Gal, have the potential to accelerate SVD by enhancing tissue calcification. Current assays to detect antibody to pig glycans are highly variable and no one assay is present to detect antibodies to all glycans (Byrne et al., 2015). The Gal antibody is detected by ELISA using a Gal tri-saccharide conjugated with bovine serum albumin. Antibodies to Sda are generally detected by agglutination assay and antibodies to Neu5Gc are optimally measured to date by glycan assays, chick anti-
Neu5Gc antibody (Reuven et al., 2016). The inconsistency amongst assays and the different conditions under which each assay is carried out makes it impossible to compare the prevalence of these antibodies in human serum in a uniform manner. I have shown that the pig kidney cell line PK15 binds to DBA and expresses B4GALNT2. This cell line also expresses the other glycans, Gal and Neu5Gc and would therefore provide an ideal substrate to assess antibody reactivity to all three glycans. Establishment of a uniform assay to detect all three glycans could potentially be used to monitor clinical immune response after BHV replacement surgery.

A uniform assay can be established by transfection with a recombinant mucin PSGL1-147 into PK15 cells, will allow for creation of a cell line PK15.16.1C which secretes high levels of 147PSGL-1IgG protein. The expression of 147PSGL1-Ig derived from this cell line will be characterised by ELISA and purification of the recombinant protein. This will allow for the detection of each of the three xenogeneic antigens, Gal, Neu5Gc and Sda on 147PSGL-11g using GSIB4, chick anti-Neu5Gc antibody and DBA respectively in ELISA and Western Blot studies. In order to measure human antibody reactivity to specific glycans, clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 nuclease methodology can be used to create mutations in PK15.16.1C cells targeting the GGTA-1, CMAH and B4GALNT2 genes. This will create specific cell lines depleted of each individual glycan (Gal-KO, Neu5Gc-KO or Sda-KO) and depleted of glycan combinations (Gal-KO, Neu5Gc-KO and Sda+) [Gal-KO, Neu5Gc+ and Sda-KO] [Gal+, Neu5Gc-KO and Sda-KO] [Gal-KO, Neu5Gc-KO and Sda-KO]). The 147PSGL1-IgG fusion protein from these CRISPR modified cell lines will be isolated and analysed. The purified protein can then be used in an ELISA assay to screen a bank of human serum of individuals exposed to pigs i.e. swine veterinarians and from normal human serum to gauge the abundance of these
anti-glycans antibodies. This will help in the development of new, longer-lasting BHVs that can be translated into clinical practice.
4.4 Summary

This project has been a unique endeavour in studying aortic valve disease by bringing the elements of genetics during development, phenotypic expression of the disease and aspects of the complications encountered with use of biological tissue valves, allowing for an in-depth and holistic understanding of a disease that continues to affect millions worldwide. Although, extensive research has been carried out on the characterisation of AVD as a congenital malformation or as an acquired disease, there was a current gap in understanding the development of the disease process across the life-course from foetus to adult. This thesis aimed at bridging this gap, by studying the developmental and disease processes as a whole from the genetics to morphological manifestation. Moreover, there were two fundamental gaps seen in the management of the disease. One, the relevance of the anatomic nature of the diseased aortic root to aortic valve repair, and two, the need for durable bioprosthetic valve tissue in cases of partial repair or replacement entirely either by surgical or interventional means. An attempt was made at narrowing this gap by looking into the structure of the aortic root and predicting its impacts on the surgical management of the aortic valve. Moreover, by testing a new strain of tissue, which proved to be unsuitable for novel bioprosthetic devices, I was able to establish that unlike other species, pigs express B4GALNT2 in endothelial cells, and no expression polymorphisms are seen in pigs, which have been the go-to source for bioprosthetic valve development. Future research could explore the use of tissues from genetically modified porcine and bovine sources for development of long-lasting BHVs. Furthermore, using the information provided by Gene Ontology, I was able to identify a number of potential genes that are involved in aortic valve development and therefore, may be affected in disease. This information, if taken further and correlated with genes involved in LV cardiomyopathies, could help
us understand whether the nature of the fused aortic valve lies in an abnormality of the LV, or if the LV remodelling is a result of the aortic valve anomaly.

In conclusion, the work carried out in this thesis shows that although the genetic mechanisms involved in aortic valve development are interlinked with one another, there are specific genetic variances responsible for the exhibition of different phenotypes of the disease. As the GO database is not capable of annotating diseases, the genetics of AVD could not be fully explored. So far, though the variances seen in phenotypic expression of AS across age groups may relate to compensatory mechanisms of the tissue for survival, they may be caused due to overlapping diseases, or different etiologic mechanisms. This suggests that aortic stenosis is a spectrum of disease across the life-course, both morphologically and genetically. Further work through gene expression and transcriptomic studies of components of developing, fully formed, and diseased aortic valves may provide a better understanding of the distinct phenotypes of disease seen across age groups, at a cellular and anatomical level. This understanding could inform the development of new interventional techniques that could steer the way to better repaired valves and help combat the difficulties encountered in current treatment practices.
“The origin and the causes of disease are far too recondite for the human mind to unravel them.”

Giorgio Baglivi; 17th Century Armenio-Italian Physician and Scientist
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