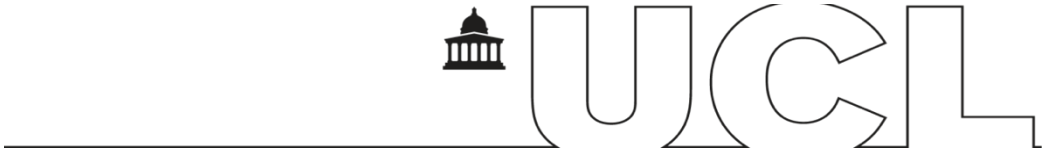

# INFLAMMATORY CYTOKINES, ENDOTHELIAL FUNCTION AND CARDIAC ALLOGRAFT VASCULOPATHY IN CHILDREN: AN INVESTIGATION OF THE DONOR AND RECIPIENT VASCULATURE AFTER HEART TRANSPLANTATION

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This dissertation is submitted for the degree of MD(Res) at University College London

February 2022

*What mankind can dream research and technology can achieve*

*Professor C. Walton Lillehei*

*We are like dwarves perched on the shoulders of giants, and thus we are able to see more and farther than the latter. And this is not at all because of the acuteness of our sight or the stature of our body, but because we are carried aloft and elevated by the magnitude of the giants.*

*Bernard of Chartes & Sir Isaac Newton*

*Where there is death there is hope*

*Professor Norman Shumway*

Declaration

I, Matthew James Fenton confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. In accordance with the University College London guidelines, this thesis is does not exceed 50,000 words.

Signed:_____

Date:_____February 28, 2020_____

Dr Matthew James Fenton MB BS, Bsc., MRCPCH

## Abstract

Cardiac allograft vasculopathy (CAV) limits the life span of paediatric heart transplant recipients. I investigated blood markers of inflammation, endothelial dysfunction and damage to both the native and transplanted vasculature in children after heart transplantation.

Serum samples were taken from paediatric heart transplant recipients for markers of inflammation and endothelial activation. The presence of systemic inflammation was assessed using serum markers including interleukin (IL) 1beta, IL 6, IL 8, c reactive protein (CRP), serum amyloid A (SAA) and tumour necrosis factor (TNF) alpha, an increase signifying the presence of a general inflammatory state. In order to assess endothelial activation measurements of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, P selectin and E selectin were taken. Each of these molecules belongs to a family of cell adhesion molecules present on the surface of endothelial cells. They are responsible for the adhesion of white blood cells and platelets to the endothelial surface and are markers of increased endothelial activation. Investigation into thrombotic mechanisms was determined by measuring Tissue Factor (TF), an important component of the coagulation cascade, von Willebrand Factor(vWF), an important protein involved in binding platelets to the site of endothelial injury and Thrombomodulin (TM), a protein involved in controlling excessive coagulation through natural anticoagulant properties and preventing inflammation. IL 10 is a cytokine with many effects on regulating the immune system and inflammation, acting to down regulate inflammation and moderating the immune response. Monocyte chemoattractant protein (MCP-1) regulates the migration and adhesion of monocytes and macrophages to atherosclerotic plaques and is present as part of a proinflammatory response. Vascular endothelial growth factor (VEGF) is involved in regulating angiogenesis, vascular permeability and inflammation and is a candidate protein involved in the development of atherosclerosis amongst other vascualar disorders.

The systemic vasculature was investigated using brachial artery flow-mediated dilatation and carotid artery intima-medial hyperplasia. CAV was investigated using intravascular ultrasound. Mean intima-media thickness (mIMT) > 0.5mm was used to define significant CAV. 48 children (25 male) aged 8 to 18 years were enrolled in the study. Patients were a median (IQR) 4.1 (2.2 to 8.7) years after transplant. Patients had increased levels of circulating IL 6 (3.86 (2.84-4.95) vs 1.66 (1.22-2.63), $p < 0.0001$*), VCAM1 (539(451-621) vs 402(342-487), $p<0.001$*), ICAM1 305(247-346) vs 256(224-294), $p=0.002$* and TM (7.1(5.5-8.1) vs 3.57(3.03-4.71,) $p<0.0001$*) and decreased

levels of TNF alpha, E selectin and P selectin, compared with controls. The systemic vasculature was unaffected.

Patients with severe CAV had raised serum von Willebrand factor and decreased serum TM. Post-transplant TM levels are elevated after transplant but significantly lower in those with mIMT > 0.5mm. TM is a protein bound to the endothelium that moderates the deleterious effects of inflammation, coagulopathy and fibrosis. vWF supports platelet to platelet adhesion and is involved in the stabilisation of clots in response to injury. This suggests that subclinical inflammation is present and natural anticoagulant/TM activity is an important area for future transplant research into CAV. This is the first time that the protective role of TM has been identified in a clinical cohort of children after heart transplantation.

## Impact Statement

Heart transplantation is the only effective treatment for end stage cardiac failure in children and adults. Whilst great progress has been made in reducing the early risk of heart transplantation, with more than 95% of children surviving 30 days after the transplant, our efforts have failed to reduce the chance of dying over the longer term.

We have had great success in preventing rejection of the transplanted heart with the help of modern medicine to protect the heart from the body's immune response. Sadly we have failed to completely understand why, years after heart transplantation, the wall of the coronary arteries, transplanted as part of the heart, thickens and eventually occludes blood supply to the heart, leading to failure of the heart and usually the death of the recipient. Occlusion of the coronary arteries or cardiac allograft vasculopathy (CAV) is the principal, generally unexplained, reason that transplanted hearts do not last as long as we expect.

On average, based on our current data, heart transplant recipients survive about 20 years after transplant. For children transplanted at a young age the impact of this severely reduced life expectancy is tragic. For example, a child transplanted at one year of age due to congenital heart disease will die as a young adult. Children are able to continue normal life after transplant, going to school, playing sports and taking a full and active part in family life. Thankfully, it is not posible to spot a child at school who has had a heart transplant as they are so well. Having a second transplant is possible but shortage of donors and adverse effects of medication over time means that only a few patients have the chance for another transplant.

This unacceptable scenario drives us to understand why transplanted hearts fail early. Why despite everything we have achieved so far we are still struggling to increase the "shelf life" of transplanted hearts. The threat of tragedy hangs over transplant families throughout their journey.

This thesis investigates important biological processes in the development of coronary artery disease after heart transplantation, attempting to affect the status quo. Whilst the cause for this disease has always been assumed to be related to attack by the immune system, other biological systems, including inflammation and blood clotting are likely to be involved.

In this thesis we have discovered that inflammation is present in transplanted children when compared to their normal peers. More importantly we have discovered that those children who have severe CAV have lower levels of a protein called TM in their blood.

TM is an interesting protein that lines blood vessels, protecting the cells from inflammation and abnormal blood clotting. It is possible that finding ways of increasing the protective effects of this “multifunctional” protein in the future will help to support an alternative process of enquiry into CAV and eventually help improve survival after heart transplantation.

## Acknowledgements

Most of all I am grateful to the amazing transplant families for inspiring me in my research efforts and for allowing me to continue help improve outcomes after heart transplantation. Despite the families' gratitude towards me and the cardiothoracic transplant team here at Great Ormond Street Hospital I am constantly amazed at their ability to continue with life after a heart transplant, with enthusiasm, fun and an incredible sense of hope for the future, no matter what this world throws at them. They are an inspiration.

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There are numerous individuals that have helped me along the way. This research has taken a long time to put together and it is the constant encouragement and support of my colleagues that has enabled it to happen, especially in the twilight of my fifth decade. Special mention to Professor Michael Burch, my mentor and friend who has always encouraged me, Dr Helen Spencer who is simply the best colleague and last but not least Dr Jacob Simmonds to whom I passed the research baton and who published his thesis well before me! I thank him for his encouragement and support picking up the real work while I indulge in this writing.

None of this is possible without the cardiothoracic transplant nurses. It takes a special individual to embed oneself in a family with chronic health conditions and their compassion, grace, poise and commitment is truly the backbone of the service. Thank you.

My boys, Freddie and Ollie who know more about hearts and organ donation than they should and give me more support than they will ever realise.

And finally my wife Dr Mette Jorgensen. I don't have enough space left on the page to extoll her academic or personal virtues so just thanks and here is to the next five decades.

Publications arising from work in this thesis

**Fenton M**, Simmonds J, Shah V, Brogan P, Klein N, Deanfield J, et al. Inflammatory Cytokines, Endothelial Function, and Chronic Allograft Vasculopathy in Children: An Investigation of the Donor and Recipient Vasculature After Heart Transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2016;16(5):1559–68.

**Fenton M**, Mahmood A, Burch M, Simmonds J, Kuhn MA. Comparative Study of Pediatric Coronary Allograft Vasculopathy Between Single Centres in North America and United Kingdom. Transplantation proceedings. 2018;50(10):3705–9.

Kuhn MA, Burch M, Chinnock RE, **Fenton MJ**. Comparison of Segmental Versus Longitudinal Intravascular Ultrasound Analysis for Pediatric Cardiac Allograft Vasculopathy. Transplantation proceedings. 2017;49(8):1899–902.

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**Fenton M**, Simmonds J, Pahl E. ISHLT Monograph Volume 13. Pediatric Heart Transplantation, Surveillance and Management of Cardiac Allograft Vasculopathy in Pediatric Heart Transplantation, Editors: Charles Canter, MD; Melanie D. Everitt, MD; Michael Burch, MD; James D. St. Louis, MD; James K. Kirklin, MD

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| | |
|---|---|
| Ab | Antibody |
| ACR | Acute Cellular Rejection |
| ADP | Adenosine Di-phosphate |
| Ag | Antigen |
| ALG | Anti-lymphocyte Globulin |
| AMR | Antibody Mediated Rejection |
| APC | Activated Protein C |
| ATG | Anti-thymocyte Globulin |
| BIT | Benign Intimal Thickening |
| CAV | Cardiac Allograft Vasculopathy |
| CD | Cluster Differentiation |
| CFR | Coronary Flow Reserve |
| cIMT | carotid Intima-medial Thickness |
| CMR | Cardiac Magnetic Resonance |
| CMV | Cytomegalovirus |
| CRP | C reactive protein |
| CT | Computerised Tomography |
| CTOT | Clinical Trials Organ Transplant |
| DNA | Deoxyribose Nucleic Acid |
| DSA | Donor Specific Antibody |
| DSE | Dobutamine Stress Echocardiography |
| E selectin | Endothelial selectin |
| EC | Endothelial Cell |

| | |
|---|---|
| eNOS | endothelial Nitric Oxide Synthase |
| FMD | Flow Mediated Dilatation |
| HDL | High density lipoprotein |
| HLA | Human Lympocyte Antigen |
| HMGB1 | Human Mobility Group Box 1 |
| ICAM | Intercellular Adhesion Molecule |
| IEL | Internal Elastic Lamina |
| IFN | Interferon |
| IL | Interleukin |
| IMT | Intima medial thickness |
| IQR | interquartile range |
| IRI | Ischaemia Reperfusion Injury |
| ISHLT | International Society of Heart Lung Transplantation |
| IVUS | Intravascular Ultrasound |
| LDL | Low density lipoprotein |
| MCP-1 | monocyte chemotactic protein |
| MHC | Major Histocompatibiliy Complex |
| mIMT | mean Intima Media Thickness |
| MMF | Mycophenolate Mofetil |
| mmHg | mm of Mercury |
| MPA | Mycophenolic Acid |
| MRI | Magnetic Resonance Imaging |
| NK | Natural killer |
| NO | Nitric Oxide |

| | |
|---|---|
| OCT | Optical Coherence Tomography |
| P selectin | Platelet selectin |
| PAR | Protease Activated Receptors |
| PHTS | Paediatric Heart Transplant Study |
| PRA | Panel Reactive Antibody |
| PSI | Proliferation Signal Inhibitor |
| PWV | Pulse Wave Velocity |
| RAGE | Receptor for Advanced Glycation End |
| RNA | Ribose Nucleic Acid |
| SAA | Serum Amyloid A |
| SMC | Smooth Muscle Cell |
| SMLC | Smooth Muscle Like Cell |
| TF | Tissue Factor |
| TGF | Transforming Growth Factor |
| TLR | Toll Like Receptor |
| TM | Thrombomodulin |
| TNF | Tumour Necrosis Factor |
| VCAM | Vascular Cell Adhesion Molecule |
| VEGF | Vascular Endothelial Growth Factor |
| vWF | von Willebrand Factor |

# 1 INTRODUCTION

## 1.1 A brief history of heart transplantation

Heart transplantation is an effective treatment for end-stage heart disease in both children and adults and is the culmination of decades of scientific research that commenced long before actual human-to-human transfer of organs could have ever been imagined.

From the late 1700's onwards progress was made in the field of immunology, with the discovery of antigens and antibodies, the discovery of blood groups and understanding of the human body's biological defenses.

By the end of the 19th century surgeons had improved their surgical skills, including suturing. At this point organ transplantation became a feature of many research laboratories. By the time the early 20th century arrived, enough was known about organ transplantation to demonstrate that cross species organ transfer, xenotransplantation, usually failed, allogenic transplants (between the same species) also failed and that autograft transplants were usually successful. It had also been appreciated that a second transplant between the same donor and recipient showed accelerated rejection. These findings highlighted our need to understand the basis of rejection of transplanted organs and demonstrated that further understanding of transplant immunology was required. Understanding the mechanisms of rejection are critical to the success of solid organ transplantation.

During the first half of the 20th century small research gains improved understanding of immunology and rejection. Seminal work by Medawar on the fate of skin grafts in mice and humans paved the way for the first solid organ transplants, and though they were unaware at the time, provided the scientific knowledge for blood group incompatible heart transplants for children in the future (1). Through the subsequent decades, rejection of the transplanted organ continued to be a problem in renal and liver transplants. Successful kidney transplants were successfully performed between identical twins. The first in the UK was performed by Woodruff in Edinburgh in 1960.(2)

From a heart transplant perspective Dr Norman Shumway, working at the Stanford Medical Centre, California, USA, with his colleague Dr Richard Lower performed extensive animal experiments between 1958 and 1965. Shumway and Lower were acknowledged as the leading pioneers in heart transplant research. By December 1959 they had successfully transplanted a heart between two dogs. Their meticulous research developed a technique to reduce the effects of warm ischaemia following procurement by cooling the heart and leaving a cuff of native atrial tissue at the back of the heart, attempting to limit the number of anastomoses required. This avoided the

need for individual anastomoses of the pulmonary veins.(3) These early experiments were crucial in preparing the world for the first human heart transplant. Among those who witnessed the experiments was Dr Christian Barnard. After a period of “dogged” research, Shumway and Lower had finally reached a point at which heart transplantation in humans might be possible.

Amidst a media storm, and with several surgeons anxious to be the first, the first human heart transplant was performed not by either Shumway or Lower or even Kantrowitz in NewYork, but by Dr Christian Barnard in Cape Town in 1967. Dr Barnard had previously been a visitor in Dr Shumway’s unit in California. The recipient lived for only 19 days and was the first in a series of heart transplants, performed both in South Africa, USA, United Kingdom and France. The success of the early pioneers pursuing heart transplant now shines before us, not least through observing the happiness and achievements of the children we have transplanted over the years. The media attention, often reluctantly, bestowed on these early pioneers was not dissimilar to the contemporary race to put the first human on the moon.

After the media storm died down it became clear that these early exploratory transplants could not be considered a success and the media of the day vilified the surgeons, reported on limited long-term survival due to rejection and a lack of suitable immunosuppressive medication. The media questioned the surgeons’ motivation and the validity of undertaking the procedure.

Despite the poor early results, two patients from the initial series of patients transplanted in South Africa lived more than ten years. This is in an era before ciclosporin where effective immunosupression was not yet available. It would seem that the “immune match” between the donor and recipient in these two cases was sufficient to enable a degree of immune tolerance. Following the poor results, the number of heart transplants rapidly declined in the first decade. Dr Norman Shumway continued to perform heart transplants and research the procedure, becoming the pioneering influence behind the success of heart transplantation.(4)

Pivotal developments occurred, allowing heart transplantation to become a successful treatment option. These included the development, by the cardiothoracic surgeon Phillip Caves, of a technique to perform an endomyocardial biopsy from the superior caval vein along with a histological classification of rejection by Dr Margaret Billingham that continues to form the diagnostic criteria for cardiac rejection diagnosis today.(5) These important developments enabled the status of the heart to be assessed after implantation and set up a process whereby the heart could be monitored and drug

responses could be assessed.

## 1.2 The development of Immunosuppression

The history of successful solid organ transplantation is closely related to the development of immune modulating medication. The broad-based immune modulating effects of steroids were the first class of drugs to be used in this regard, however it was not until the discovery and development of agents capable of interfering with the rejection process, through direct effects on lymphocytes, that heart transplantation became successful enough to provide a more realistic treatment option. The first of these agents to be discovered was a less toxic derivative of 6 mercaptopurine called azathioprine. This purine analogue interfered with the proliferation of lymphocytes, producing an immunosuppressant effect. Azathioprine in combination with steroids significantly improved post-transplant survival, particularly in kidney transplants, but with only 50% survival at a year the outcomes remained poor.

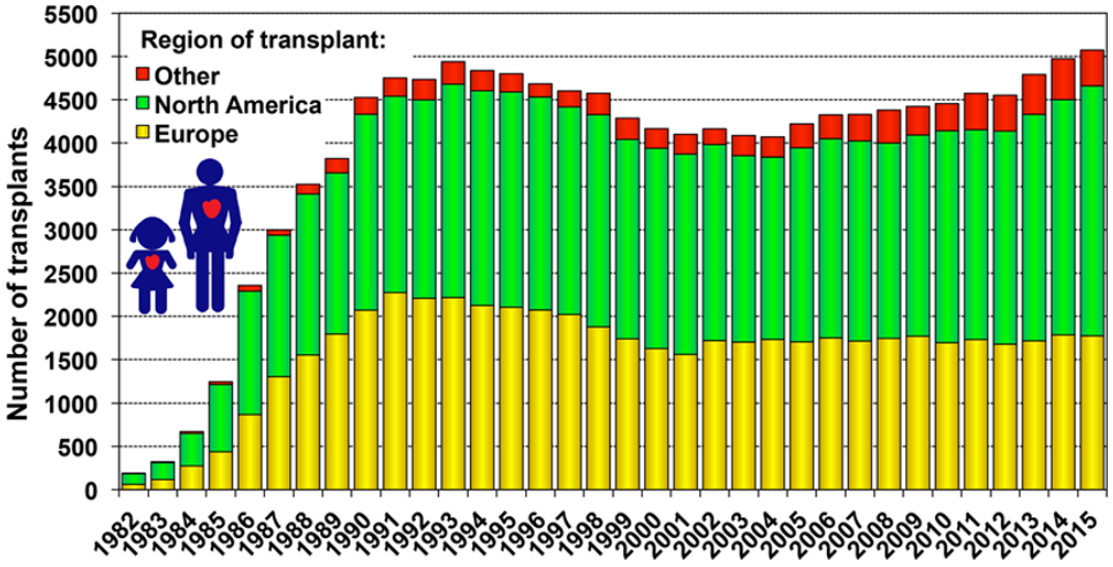


**Figure 1.1 Number of heart transplants (adult and paediatric) by year and geographic region. Reproduced from Lund et al.(6)**

The major breakthrough for solid organ transplantation came with the discovery of the calcineurin inhibitor ciclosporin in 1978. Its action as a potent immunosuppressant improved transplant outcome, and the number of heart transplants increased significantly from that point on. The beneficial actions of ciclosporin are offset by its significant renal toxicity, potential to cause diabetes and compliance issues due to hypertrichosis. More recently tacrolimus, a calcineurin inhibitor that inhibits the FK506 binding protein, is the preferred agent used to prevent organ rejection. Tacrolimus is reported to cause less renal toxicity though may be more diabetogenic. It has a tendency to cause less hypertrichosis and as a result improves compliance in the

paediatric population, critical to the prevention of graft rejection.

Most transplant protocols will use a combination of agents to prevent rejection, with azathioprine continuing to be an adjunctive agent. More recently azathioprine has been replaced with mycophenolic acid (MPA), given in the form of mycophenolate mofetil (MMF). This blocks the enzyme responsible for the production of guanosine nucleotides and as a result prevents lymphocytic proliferation by impairing DNA synthesis. Other cells have salvage pathways for this enzymatic process and as a result only lymphocytes are affected, leading to immune modulation and prevention of rejection. Careful monitoring of white blood cell numbers is required to prevent lymphopenia and toxic side effects in the form of gastrointestinal upset are relatively common.

The immune attack following heart implantation is at its greatest immediately following implantation, and as a result many transplant centres around the world will use induction therapy either just before or at the time of reperfusion of the allograft. This more aggressive attempt to prevent rejection is focused on rendering lymphocytes inactive, preventing the possibility of immediate or early rejection. These agents are given intravenously so as to have maximum effect. Steroids, as methylprednisolone, are used due to the wide effect on inflammation and the immune system and continue to be an important agent in the prevention of rejection. More targeted monoclonal or polyclonal antibody treatment is also used to impair lymphocytic activity and prevent rejection. Antibodies to both lymphocytes (antilymphocyte globulin - ALG) and thymocytes (antithymocyte globulin – ATG) are used and were initially produced by inoculating horses or rabbits with human lymphocytes or thymocytes and harvesting the antibodies. Recently a monoclonal antibody to the CD25 receptor on the alpha chain of the interleukin 2 receptor of activated T lymphocytes has been developed (basiliximab) and targets those cells responsible for allorecognition and initiation of the rejection response. Other monoclonal induction agents targeting both T and B cells, alemtuzumab, are also being investigated, however a careful balance needs to be maintained between preventing rejection and rendering the recipient susceptible to life threatening sepsis early after transplant. It is also worth commenting that universal acceptance of a single approach to antirejection therapy after heart transplantation has yet to emerge and that even the use of induction agents is not fully adopted worldwide. In the most recent report from the International Society of Heart Lung Transplantation only 50% of centres include an induction agent in their drug protocol.

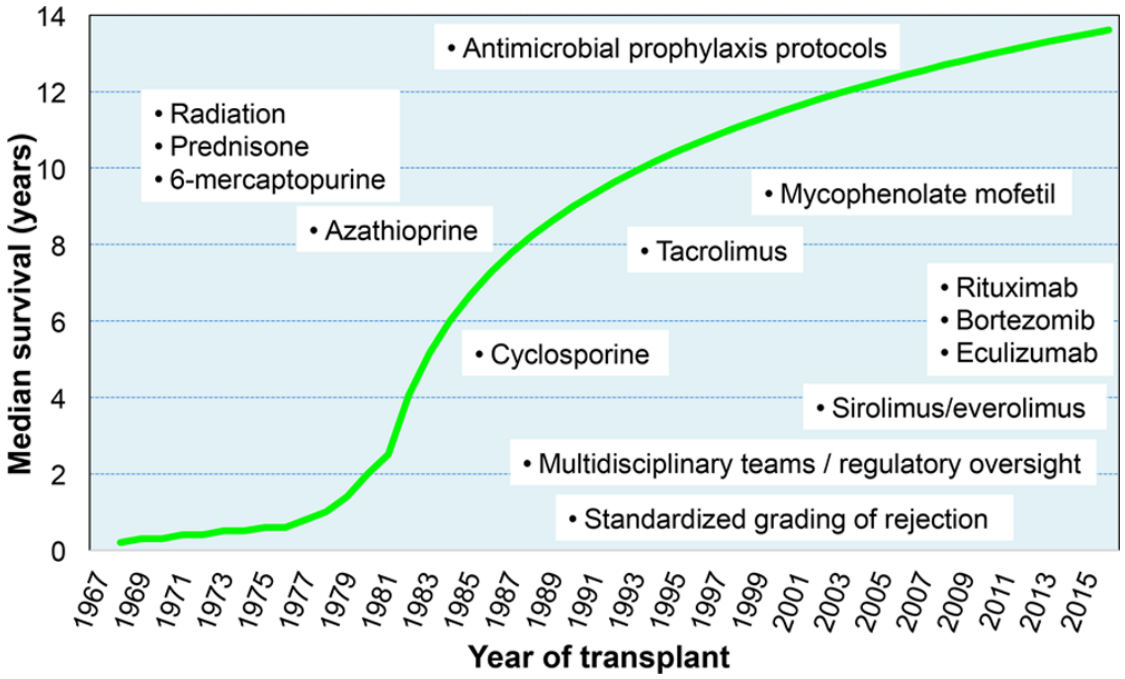


**Figure 1.2 Median survival after heart transplantation and approximate time of introduction of key immunosuppressive agents; standardized clinical care approaches in heart transplantation based on data submitted to the International Society for Heart and Lung Transplantation. Reproduced from Stehlik et al. (2018) (7)**

## 1.3 Outcomes after heart transplantation in children

Outcomes after transplant have continued to improve with a significant era effect. Improvement in operative and perioperative techniques have significantly improved surgical mortality and life-threatening rejection is now extremely rare, confined primarily to those patients who have pre-formed antibodies directed specifically to the donor organ. The improvement in outcomes can be seen in Figure 1.3. Whilst these results are excellent it is important to note that the slope of the survival curve has the same gradient a year after transplantation. This demonstrates that the benefits are primarily within the first year after transplant and that outcomes conditional on surviving the first year do not show a significant difference in mortality over time. The medium to long-term mortality rates have not changed over time.

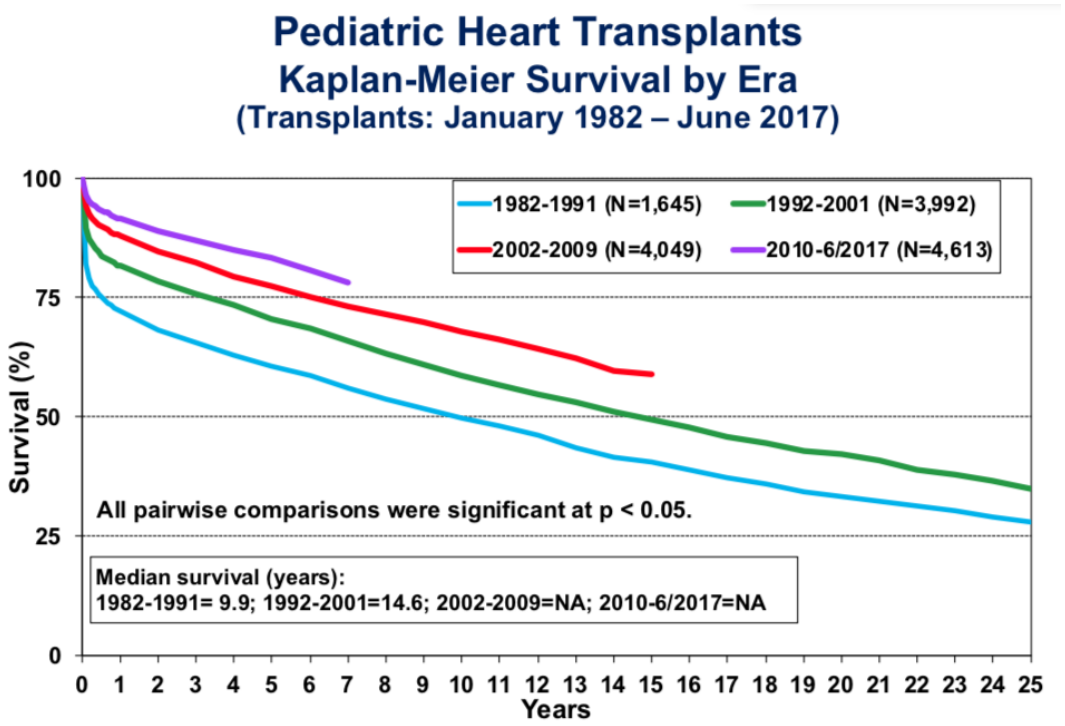


**Figure 1.3 Paediatric Heart transplants Kaplan-Meier Survival by Era (January 1982 - June 2017). Reproduced from Rossano et al. JHLT (2019) (8)**

Causes of death in patients differ depending on time post-transplant with rejection and graft failure being the most common cause of death within the first 30 days. After this coronary artery disease, in the form of cardiac allograft vasculopathy (CAV), is the most important cause of death 5 years after transplant and is the focus of this thesis.

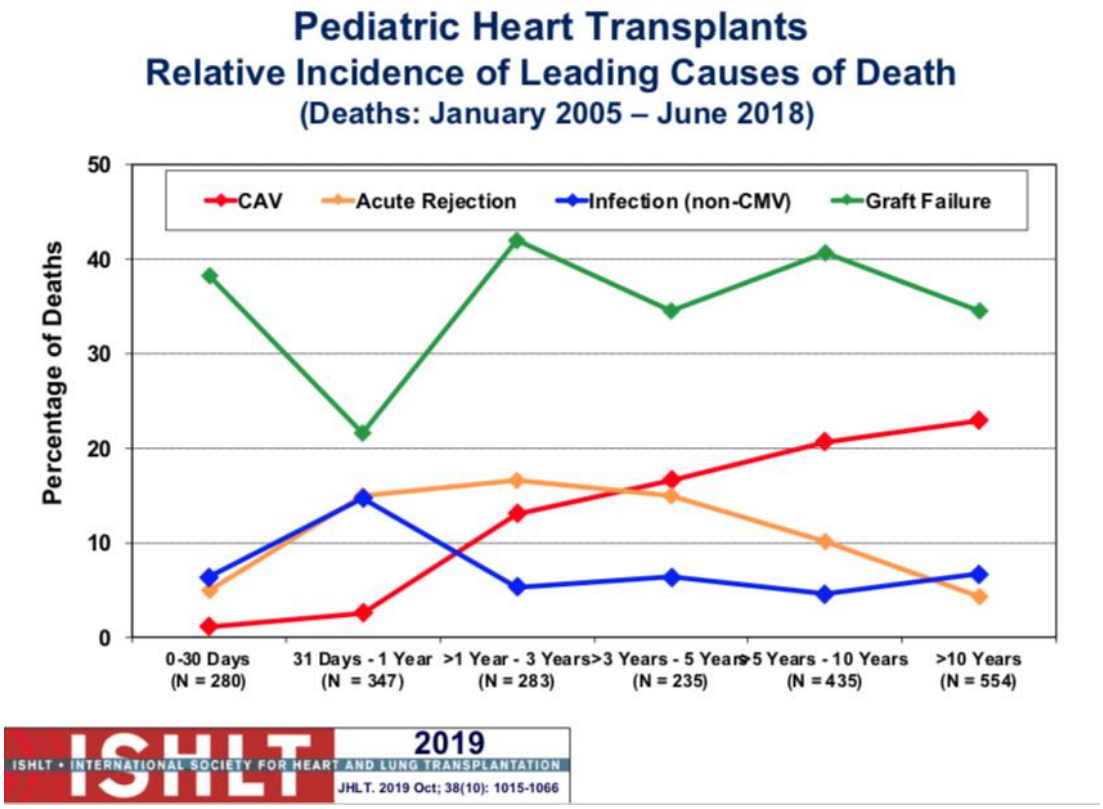


**Figure 1.4 Relative Incidence of Leading Causes of Death (Jan 2005 - June 2018). Reproduced from Rossano et al. JHLT (2019) (8)**

## 1.4 Cardiac Allograft Vasculopathy (CAV)

CAV is a ubiquitous process after heart transplantation, leading to progressive occlusion of mainly the epicardial coronary arteries in both adults and children. CAV is one of the most important causes of graft loss and mortality after transplant accounting for more than 16% of deaths from three years post-transplant. CAV is likely to be a contributing factor in chronic graft failure, a non-specific registry-based diagnosis. Freedom from CAV is 40% by 17 years. (Figure 1.5)

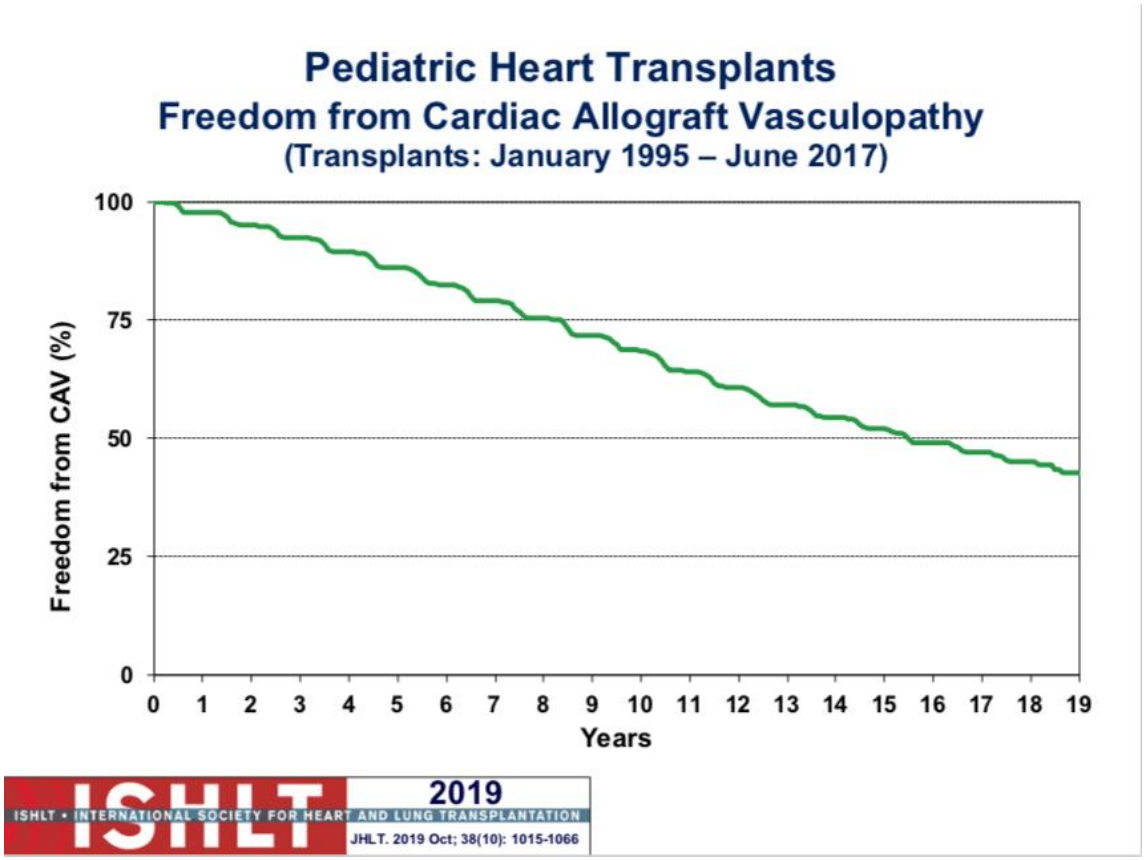


**Figure 1.5 Freedom from Cardiac Allograft Vasculopathy. (January 1995 - June 2017) Reproduced from Rossano et al. JHLT (2019). (8)**

Once CAV is diagnosed, short-term mortality rates are high with between 60-70% of recipients experiencing graft loss by 2 years post CAV diagnosis. (Figure 1.6)

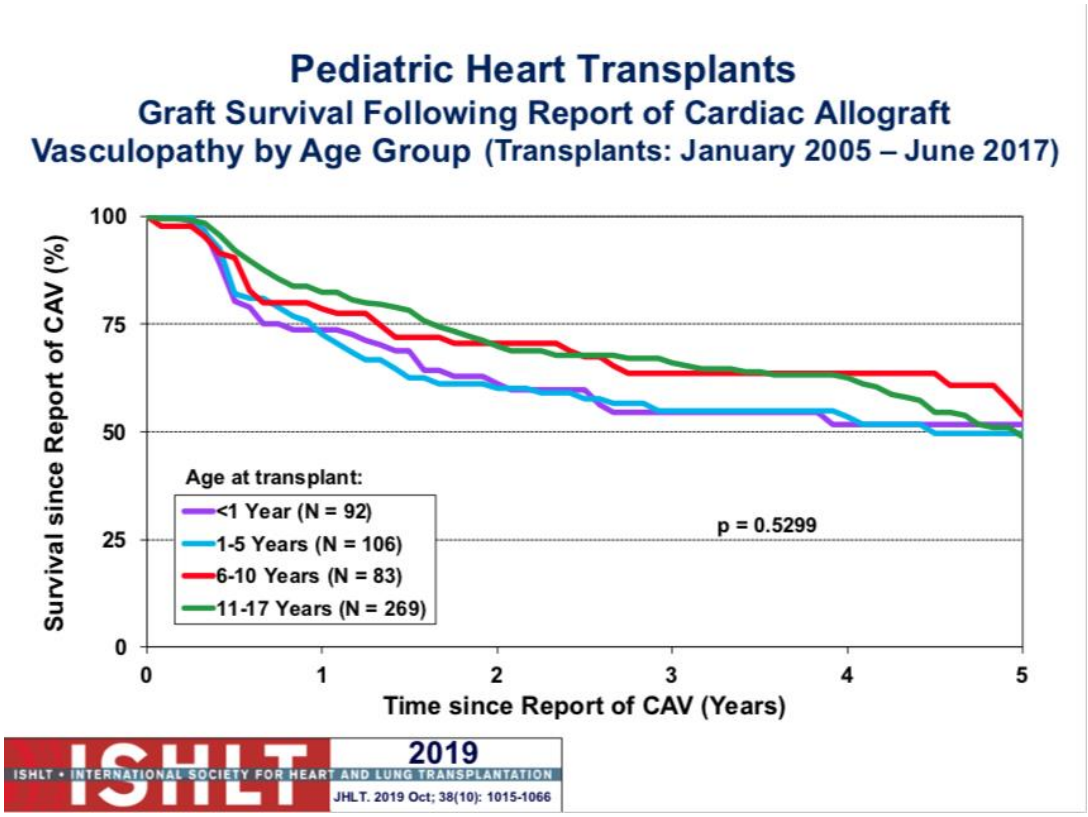


**Figure 1.6 Graft survival following report of CAV by age group. Reproduced from Rossano et al. JHLT (2019). (8)**

### 1.4.1 Histology and pathogenesis of CAV

CAV is characterized by diffuse thickening of the coronary artery wall and has a distinct appearance when compared to that of atherosclerosis seen in the general population. Progressive thickening of the intimal layer leads to luminal stenosis, causing obstruction to blood flow and tissue ischaemia. Intimal thickening is a normal process in human coronary arteries and increases with age.(9) This benign intimal thickening (BIT) can be observed in normal autopsy specimens and the smooth muscle cells present can explain the source for the proliferation of smooth muscle cells characteristic of CAV.(10) In CAV a neointima is present and is made up of loose connective tissue, smooth muscle cells and mononuclear cells. This neointima is covered by endothelial cells. The neointima sits on the luminal side of the smooth muscle cells that have already developed prior to transplant with an internal elastic lamina (IEL) beneath, adjacent to the adventitia. As the neointimal layer increases in size there is remodeling of the vessel wall in order to maintain luminal area. The increase in vessel size aims to preserve continuity of the lumen. Once the limit of remodeling is reached due to either spatial constraint or reaching the elasticity limit of the internal elastic lamina, increasing thickening leads to increasing reduction in luminal area and stenosis develops.(11–13)

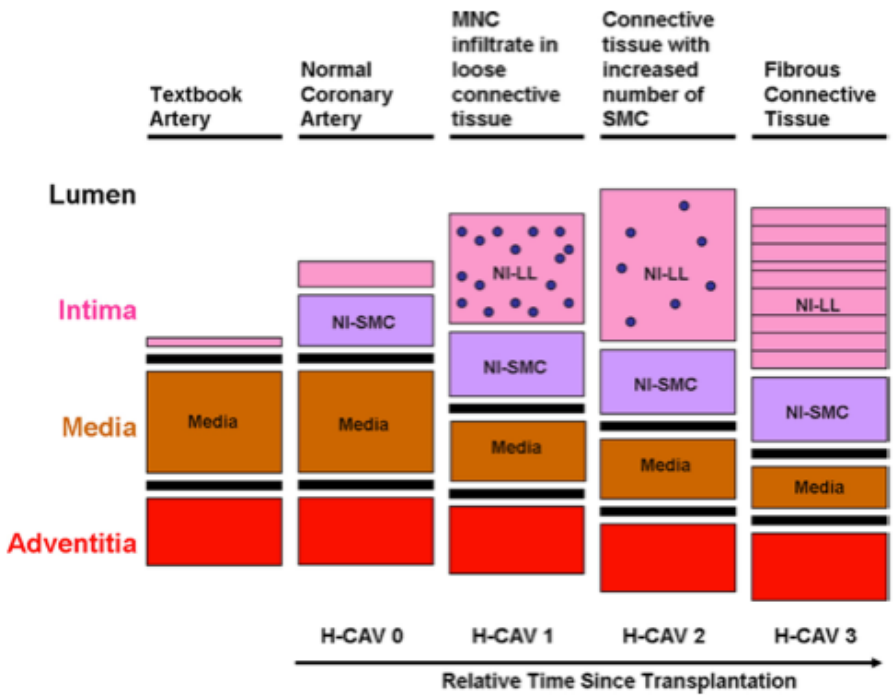


**Figure 1.7 Schematic presentation of histological layers for CAV. The left panel demonstrates the coronary artery of a newborn. Changes in vessel wall architecture are displayed as relative in time since transplant and H-CAV type. Early after transplant the NI-LL is infiltrated by T-Lymphocytes as a consequence of rejection. In reaction to this infiltration loose connective tissue becomes more solid and eventually results in a fibrous layer. After a peak in NI thickness and luminal narrowing this process is reversed. As the media progressively gets thinner over time, the SMV layer seems relatively spared. H-CAV = histological cardiac allograft vasculopathy. NI-LL neo intima luminal layer, NI-SMC = neo intima smooth muscle cell layer, MNC = mononuclear cell. Reproduced from Huibers et al Atherosclerosis 2014.(13)**

Despite the recognition of CAV as a significant problem, a cohesive pathogenic mechanism for its development has remained elusive. Investigations into its aetiology have led to a hypothesis that involves both immune and non-immune factors. Bearing in mind the presence of immune cells at the luminal and abluminal borders of CAV lesions and the relative normality of the recipient vasculature, a primarily immune-mediated injury is likely to be the predominant cause.

### 1.4.2 CAV and the immune response

The endothelial cells of the donor cardiac allograft are the first to make contact with the recipient immune system. Recognition of foreign endothelial cells, with non-self human leukocyte antigens, leads to direct dendritic cell (antigen presenting cell) identification of foreign major histocompatibility complex (MHC) molecules on donor cells, indirect allorecognition via internalisation and presentation of processed antigens and a semi-direct route involving acquisition of MHC molecules through direct dendritic cell contact.

This donor/host interaction triggers an immune response, likened to an ineffective delayed type hyperactivity response against endothelial cells (EC) and smooth muscle cells (SMC) in the donor vasculature.(14)

The two processes of direct and indirect allorecognition cause activation of T cells and possibly the formation of donor specific antibodies (DSA). Production of cytokines follow, particularly IL 2, interferon-gamma and Tumour Necrosis Factor alpha. The mammalian target of rapamycin (mTOR pathway) is stimulated by DSA and stimulates endothelial and smooth muscle cell activation.

The pathways involved in smooth muscle cell proliferation and protein synthesis are reported to be via the p70 ribosomal S6 kinase and eukaryotic initiation factor 4E-binding protein.

Whilst this response is not effective enough to completely eradicate the foreign cells the damaged cells are able to trigger events that leads to proliferation of smooth muscle cells and progressive occlusion of the lumen.(15) These damaged cells can become an ongoing target for an alloimmune response. However, the trigger for a chronic response does not require a persistent alloimmune component. The response to injury theory of CAV suggests that other cells and pathways triggered by the alloimmune response can be persistent, particularly marcophages, producing ongoing damage which can be exacerbated by non-immune factors such as ischaemia-reperfusion, viral infections such as cytomegalovirus (CMV) and metabolic factors such as hyperlipidaemia. (16)

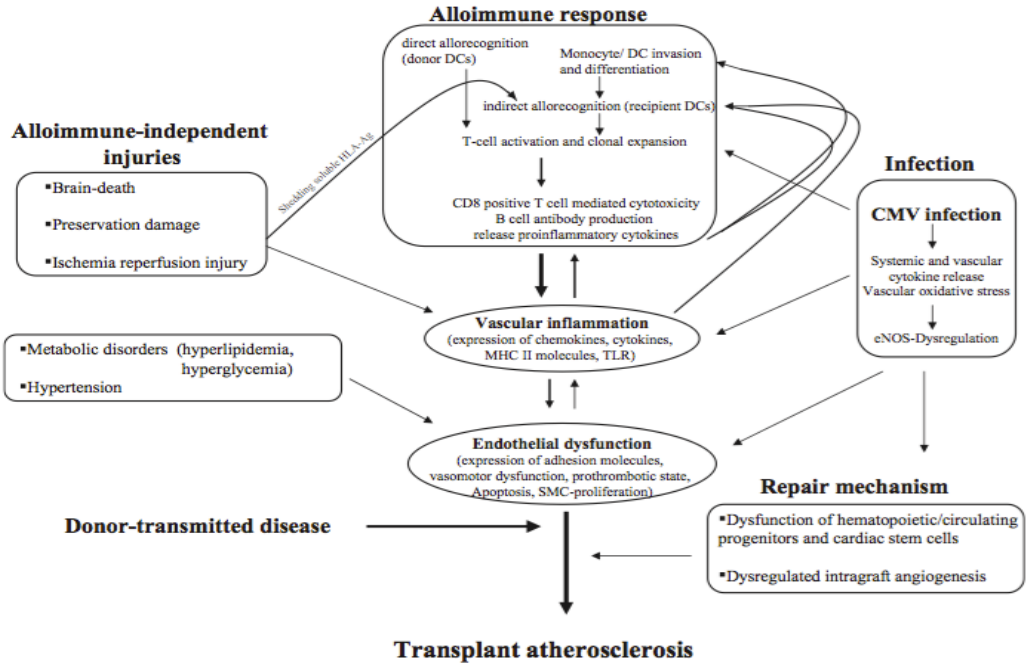

**Figure 1.8 Risk factors for the development of CAV. Collaboration and interaction of alloimmune-dependent and -independent factors influencing the pathogenesis of transplant vasculopathy. Ag indicates antigen; CD, cluster of differentiation; eNOS, endothelial NO synthase; and SMC, smooth muscle cell. (From Cardiac allograft vasculopathy: recent developments. Schmauss et al. *Circulation*. 2008. (71) 5**

The innate immune system provides the initial immune response at the time of donor organ reperfusion. The release of Heat Shock Protein 70 (HSP70) and High Mobility Group Box 1 (HMGB1), in response to Ischaemia Reperfusion Injury (IRI), signals leukocytes through Toll like receptors (TLR4) to produce IL 6 and TNF-alpha. This then leads to activation of cells involved in both the innate and adaptive immune response.(17)

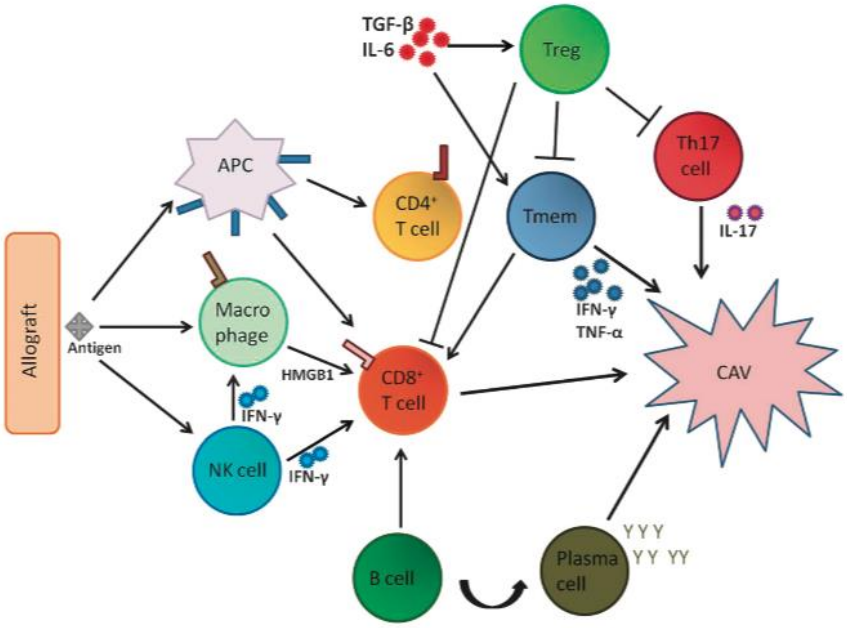


**Figure 1.9 Immunological mechanisms in CAV.** Overview of the most important immune cells involved in the development of CAV. The transplanted heart (allograft) is recognized by activated presenting cells (APC), macrophages, and natural killer (NK) cells. The foreign antigen is taken up by APCs and presented to $CD8^+$ and $CD4^+$ T cells. Macrophages and NK cells take up antigen and attack the allograft directly and/or stimulate $CD8^+$ T cells. Produced cytokines induce Treg cells and Tmem cells. The Treg cell inhibits the activation of Th17 and Tmem cells. B cells do not only differentiate into plasma cells to make antibodies but also support $CD8^+$ T cells. Th17, Tmem, $CD8^+$ T cells together with antibodies can lead to rejection of the transplanted heart. APC, antigen-presenting cell; Tmem, memory T. (Reproduced from Jansen et al; Transplantation 2015)(17)

Research has shown that the presence of IFN gamma (IFNγ) is an important mediator in the development of CAV. Mice where IFNγ has been blocked by antibody or genetically removed do not develop CAV.(18–20) The precise mechanism for this remains elusive, however it is known that IFNγ has pleiotropic effects on a wide variety of immune effector and helper cells, leading to a diverse cytokine, chemokine, adhesion molecule and extracellular matrix response. IFNγ can also be produced by macrophages and by smooth muscle cells recruited into the lesions, directly creating a

positive feedback loop, leading to persistent activation, migration and proliferation of smooth muscle cells and development of CAV.(19)

Early studies of CAV assumed that SMC infiltration into the vascular wall were derived from the muscle cells present in the media layer of the donor vasculature. It has now become clear that a large proportion of these cells are host derived; from both the bone marrow and from circulating host precursor cells. It has also become clear that the SMC that infiltrate the CAV lesion have different cell signalling properties to the SMC that are normally part of the anatomy of the vascular wall. This has led researchers to refer to these pathological SMC as smooth muscle like cells (SMLC), an important distinction, allowing us to differentiate between the two distinct cell populations. If a suitable target for therapy is to be identified then it is the pathological SMLC that needs to be affected by pharmacological blockade.(21)

In our search for a therapeutic target it has become increasingly clear that we acquire a thorough understanding of the cell interactions at play in the development of CAV. These extremely complex and interwoven pathways might help to enhance our understanding and identify a target for future therapy.

It is not just activated lymphocytes and macrophages that are capable of signalling SMC recruitment but also EC's and SMC's themselves that are a source of ligands (RANTES, MCP-1, (MIP)-1) capable of stimulating chemokine receptors. By this mechanism damaged cells themselves can become part of the feedback loop recruiting SMLC's even in the absence of an alloimmune response.

### 1.4.3 The role of adhesion molecules

It is clear from current research that SMLC proliferation, the hallmark of the CAV lesion, arises from both the luminal and the abluminal surface of the media. For inflammatory cells to enter the vascular wall adhesion molecules are required to enable effector cells to be recruited and migrated to the site of injury. Endothelial cell adhesion molecules are responsible for this process and enable the adhesion and firm attachment of inflammatory cells to activated endothelium. E and P selectins provide a weak attachment between the EC membrane and circulating cells to enable rolling along the endothelial surface before firm adhesion is provided by ICAM-1 and VCAM-1. It is therefore not surprising that EC expression of these molecules correlates with both acute rejection and CAV.(22,23) Attempts to "therapeutically" interfere with adhesion molecule interactions has been disappointing with respect to preventing rejection or CAV, though some success has been shown in preventing CAV in ICAM-1 deficient grafts.(24)

### 1.4.4 The role of antibody and B cells in the development of CAV

Along with T cells, B cells are also important in the development of CAV. B cells and subsequent plasma cells are able to produce antibodies which can damage smooth muscle cells in the transplanted coronary arteries.(25)Once the antibodies are present they attack the donor graft endothelial cells and activate the complement cascade. The complement cascade can activate many cell types and lead to fibrosis and CAV.

It is also interesting that B cells are able to promote CAV without the presence of detectable antibody in the serum. B cells can stimulate T cells via costimulation and cytokine production.(25,26)

Clinically, severe CAV development is associated with increasing human leucocyte antigen (HLA) mismatch, as unlike renal transplantation we are unable to match on the basis of HLA type. The presence of preformed antibodies to donor HLA, as is common in patients who have become sensitised by previous blood transfusions, surgery with human materials or previous pregnancy is associated with worse post-transplant outcomes and more severe CAV. In children transplanted for congenital heart disease the presence of these preformed antibodies needs to be avoided if possible, making the procurement of a suitable donor naïve for the specific antigens difficult. The development of de novo donor specific antibodies is associated with graft failure after transplantation and worse outcome.(27)

The role of antibody after heart transplantation is primarily related to the presence of antibody mediated rejection (AMR). AMR has been a poorly defined entity and was first described by Herskowitz.(28) Initially AMR developed as a diagnosis of exclusion in circumstances where allograft dysfunction was present in the absence of cellular rejection. Over the last ten years successive consensus statements have been published defining the histological and immunopathological features required to diagnose AMR. Clinical allograft dysfunction is required with histological evidence of acute capillary injury characterised by endothelial changes or the presence of macrophages in the capillaries.

The mechanisms by which B cells and antibodies are implicated in the development of CAV are not clear as yet but the association between the presence of de novo DSA production and CAV development implicates B cells and antibodies in CAV pathogenesis.(27)

### 1.4.5 The role of IL 17 and Th17 cells

Il-17 has always been known to be associated with the development of CAV. The role

of IL 17 is as an inflammatory cytokine which increases cell adhesion molecule expression on smooth muscle cells. An interesting paradox is present in that Th17 cells are induced by transforming growth factorT(GF)-beta which also induces regulatory T cells that moderate the inflammatory response.(17)

### 1.4.6 The role of the innate immune response

The success of heart transplantation has involved the development of immunosuppressive agents that primarily act to control the adaptive immune response, interfering with T cell numbers and recruitment and preventing cell mediated immunity. Whilst the focus on suppressing the adaptive immune response has been successful in prolonging life after transplant, immunosuppressive medication continues to be required and we are unable to influence chronic graft loss due to graft failure and CAV. The innate immune response is an important component of our body's immune defences and reflects the immediate response to an immunological threat. This response is of course the most useful when pathogens such as viruses or bacteria breach mechanical defences. In solid organ transplantation this is an unwelcome response leading to tissue damage early after transplant, instigating a process that is likely responsible for the graft damage that continues throughout the life of the graft and in the case of heart transplantation the recipient themselves. Recent research into the role of the innate immune response, particularly natural killer cells (NK), Toll like receptors (TLRs) and complement deposition warrants further discussion and provides further insight in our search for therapeutic interventions to prevent the initiation and development of CAV.

NK cells are the principal agent involved in invoking the innate immune response. NK cells are capable of initiating a response to target cells without having prior sensitisation. They are activated by cells who lack self MHC, causing target cell lysis and cytokine release, principally IFN$\gamma$. Until recently the role of NK cells in acute rejection was not fully appreciated but experiments in mice models have demonstrated their role in causing rejection under special circumstances. The most convincing evidence for their role in the development of CAV comes from a heart transplant mouse model involving cardiac transplant from parents to their F1 hybrid progeny. Grafts implanted through this genetic lineage are accepted by the recipient, avoiding activation of the acquired immune response and isolates the innate immune response. Cardiac grafts were accepted indefinitely as would be expected in the absence of immunosupression, however autopsy studies at 56 days post implantation demonstrated severe CAV in 19 out of 22 organs. Repeating the experiment from parents to F1 hybrids who were deficient in IFN-$\gamma$ prevented the development of CAV.

This strongly implicates the development of CAV through an NK/interferon mediated pathway. Further information relevant to an in vivo reponse in humans remains elusive.(29)

Toll like receptors are an ancient group of transmembrane proteins present on a wide variety of cells, many of which are involved in heart transplant rejection. These include epithelial cells, antigen presenting cells, macrophages and both T- and B- lymphocytes. Particularly interesting from a transplant perspective is that ligands release during IRI are able to activate TLRs. The endogenous protein hyaluronan, messenger molecules high mobility group box 1 and heat shock protein 70 are all released following IRI. The consequences of toll like receptor (TLR) activation is an inflammatory response, increased production of IL 1, IL 6 and TNFalpha and its associated effect on cardiac impairment.(30) Evidence for the long term effects of TLR activation comes from experiments suggesting that TLR signalling prevents the development of tolerance and attenuates the accumulation of T reg cells in cardiac allografts.(31)

Complement and its activities is responsible for removing cellular debris and plays a crucial role in eliminating invading microorganisms, protecting the tissue form damage. The complement pathways play a crucial role in the innate immune response and as part of its stepwise approach is capable of a graduated response, commensurate with the severity of the threat. In addition to being an effector of the immune response it is also involved in control and recruitment of co components of the immune response. As a result complement plays a role in homeostatic mechanisms. Crucial for understanding the regulatory role of complement is the understanding that complement has different roles depending on its binding to a self or non-self cell membrane surface. Complement (and coagulation) protease enzymes are distributed via their inactive form (zymogen) and require local activation before they become active. This ensures time and site-specific activity. The nine central components of the complement cascade (C1-C9) are complemented by an ever-expanding number of control proteins. Following heart transplantation complement genes are upregulated in leukocytes in the presence of rejection. Much of the interest in the role of complement activation has been focused on the role IRI has on activating the complement pathway. IRI is capable of triggering a response enabling complement to bind to the graft endothelium, causing inflammation, coagulation and permanent tissue injury. C4d and C3d deposition on several serial biopsies early after transplant are associated with IRI and is related to increased episodes of rejection on later biopsies.(32) C4d deposition is also linked to the development of CAV as detected using intravascular ultrasound (IVUS) in the first year after transplant.(33)

This evidence suggests that innate immunity plays a significant role in the development of rejection and CAV after heart transplantation and that our current immunosuppressive regimes, whilst mostly adequately suppressing acquired immunity does not deal with components of innate immunity, particularly early after transplant. Failing to adequately suppress NK cell and IFNγ is likely to set up the donor coronary vasculature to develop CAV in the future.

### 1.4.7 Risk factors for the development of CAV

Older donor age, older recipient age, donor cigarette use, recipient black race, transplant era, no induction therapy, rejection in the first year post-transplant and increasing number of rejection episodes have all been linked to progression of CAV. (34,35) In the paediatric setting, increased graft loss was predicted by adding echocardiographic and hemodynamic data to the International Society of Heart and Lung Transplantation (ISHLT) grading system. (36)

The presence of anti HLA antibodies is known to be detrimental to graft survival in paediatric patients. Those patients with a panel reactive antibody (PRA) >10% are more likely to have early onset graft vasculopathy.(37) Survival and graft loss is influenced by DSA and compounded by the presence of de novo DSA, particularly to Class II antigens.(38,39) In particular those patients with DSA were more likely to develop CAV, especially if the antibodies were persistent. (38,40) Class II HLA-DR antigens are expressed on donor endothelial cells and are a key trigger for the immune response to the allograft. An increase in donor specific and non-specific HLA antibodies is associated with a poor outcome after transplant and is implicated in the development of CAV. (41)

## 1.5 Surveillance and diagnosis of CAV

As we have discussed CAV is a histological diagnosis characterised by progressive luminal loss in the transplanted coronary arteries. The disease is not isolated to the epicardial vessels but involves the cardiac allograft vasculature (aretries, veins and capillaries) and can be identified at myocardial blood vessel level. Clinically CAV has come to be defined by the progressive luminal narrowing seen in the large conduit arteries that can be imaged using coronary angiography.

### 1.5.1 Methods of diagnosis and surveillance

#### 1.5.1.1 Coronary angiography

Invasive coronary angiography remains the most frequently used method for assessing

CAV. There is no consensus on the periodicity that angiography should be performed but varies generally between annual or biennial studies. Given the diffuse thickening of the coronary wall characteristic of this disease, this technique provides only “lumenography”, and as a result lacks the sensitivity required to detect the presence of early disease and progression early after transplant when the pathological process is likely to be at its most active. (42,43)

ISHLT have published a guideline on the diagnosis of CAV and this has subsequently been adapted for paediatric patients. (36,44) The current grading system focuses on disease in epicardial vessels with an increase in severity based on a functional assessment of the allograft. The presence of more peripheral small vessel disease is more likely to cause deterioration in overall function, causing reduction in systolic and/or diastolic function, with the development of restrictive physiology in more severe cases. In general, the grading system is rarely used to report severity and a lack of standardization across both adult and paediatric studies is partly responsible for the lack of cohesiveness in adopting a universal reporting strategy. Previously, Pahl et al addressed this by using a simplified classification of CAV to provide standardization in reporting, without which reports lack the required homogeneity to determine progression of disease. (35)

**Table 1 Recommended ISHLT nomenclature for cardiac allograft vasculopathy.**

**International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. Mehra et al JHLT 2010. (16)**

| **Recommended nomenclature for cardiac allograft vasculopathy** | |
|---|---|
| ISHLT CAV0 (not significant) | No detectable angiographic lesion |
| ISHLT CAV1 (mild) | Angiographic left main (LM) <50%, or primary vessel with maximum lesion of <70%, or any branch stenosis <70% (including diffuse narrowing) without allograft dysfunction |
| ISHLT CAV2 (moderate) | Angiographic LM <50%; a single primary vessel ≥70%, or isolated branch stenosis ≥70% in branches of 2 systems, without allograft dysfunction |
| ISHLT CAV3 (severe) | Angiographic LM ≥50%, or two or more primary vessels ≥70% stenosis, or isolated branch stenosis ≥70% in all 3 systems; or ISHLT CAV1 or CAV2 with allograft dysfunction (defined as LVEF ≤45% usually in the presence of regional wall motion abnormalities) or evidence of significant restrictive physiology |
| Definitions | a). A “Primary Vessel” denotes the proximal and Middle 33% of the left anterior descending artery, the left circumflex, the ramus and the dominant or co-dominant right coronary artery with the posterior descending and posterolateral branches.<br><br>b). A “Secondary Branch Vessel” includes the distal 33% of the primary vessels or any segment within a large septal perforator, diagonals and obtuse marginal branches or any portion of a non-dominant right coronary artery.<br><br>c). Restrictive cardiac allograft physiology is defined as symptomatic heart failure with echocardiographic E to A velocity ratio >2 (>1.5 in children), shortened isovolumetric relaxation time (<60 msec), shortened deceleration time (<150 msec), or restrictive hemodynamic values (Right Atrial Pressure >12mmHg, Pulmonary Capillary Wedge Pressure >25 mmHg, Cardiac Index <2 l/min/m2) |

### 1.5.2 Intravascular Ultrasound (IVUS)

IVUS is a technique performed in conjunction with angiography that allows a small ultrasound probe to be placed inside the coronary artery via a guide catheter. The circumferential nature of the images acquired allows visualisation of the intima-medial layer and distinguishes this from the adventitia. Early studies using IVUS to investigate the severity of intima-medial thickening developed the Stanford Score to express the severity of CAV observed using IVUS.(Table 2)

**Table 2 Stanford grade classification for IVUS description of CAV**

| | Class | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Severity | Minimal | Mild | Moderate | Severe |
| Intimal thickness | <0.3mm | >0.3mm | 0.3 to 0.5 mm OR | > 1.0mm OR |
| | $<180^0$ | $>180^0$ | >0.5mm, $<180^0$ | >0.5mm, $>180^0$ |

This score reflected the most severely affected segment of the allograft arteries and as a result much of the data reflecting changes along the length of the vessel were ignored.(45) More recently we have used an automated pullback technique, acquiring a longitudinal view and allowing measurement of vessel characteristics including lumen and vessel areas, intima-medial thickening and assessment of stenosis. The additional benefit of this technique is that by using fiduciary markers, such as vessel branching, identical segments of the coronary artery can be imaged overtime and a reproducible and accurate assessment of baseline disease and progression can be used. This technique has been developed by me as part of this thesis and a description of this will be included in the methodology. IVUS studies have become the gold standard for research projects into CAV though its invasive nature and prohibitive costs have meant that it has failed to become established as a technique in the majority of adult cardiac transplant centres and in only a few paediatric ones.

IVUS has been used clinically to identify a high-risk group post transplant. Patients with an increase in intimal medial thickness (IMT) greater than 0.5 mm within the first year are at a significantly higher risk of cardiac events at five years post transplant.(46,47) This has identified a high risk group for use in further clinical studies investigating risk factors and biological mechanisms, including defining the cutoff for significant CAV included in this thesis.

### 1.5.3 Optical coherence tomography (OCT)

Since the development of IVUS over twenty years ago invasive imaging techniques have continued to develop. OCT is a technique for obtaining sub-surface images of translucent or opaque materials at a resolution equivalent to a low-power microscope. Medical devices are now available to use inside the coronary artery and has a significantly better resolution than IVUS as the technique is based on light rather than

sound waves. There are pros and cons to using this technique in preference to IVUS and it is likely that the techniques will remain complementary to each other. OCT allows high resolution images 1-2 mm below the endothelial surface. The improved resolution makes it possible to distinguish between the intima-medial layers, likely to allow us to improve our understanding of the pathogenetic principles in real time after transplant.(48–50) IVUS and OCT remain limited to the investigation of epicardial coronary vessels.

### 1.5.4 Functional assessment of the microvasculature: Coronary Flow Reserve (CFR)

The effects of CAV on the microvasculature remain unresolved using these 2D imaging techniques. Coronary flow reserve (CFR) has been used to assess the microvasculature in patients with atherosclerosis and has been extended to heart transplant patients in diagnostic studies of CAV.(51,52) The technique involves measuring coronary blood flow using a Doppler flow wire as baseline and then again following intracoronary infusion of agents active on the coronary vasculature. The increased flow in response to these agents is expressed as a ratio with baseline flow providing an assessment of the microvasculature and its vascular function. Most commonly adenosine has been used due to its short half-life and ability to act on adenosine di-phosphate (ADP) receptors to cause vascular dilatation. Other agents have been used including acetylcholine and substance P to assess endothelial induced vasodilatation. CFR = hyperaemic flow/baseline flow.

This technique is used in a handful of paediatric centres, though is a valid attempt at assessing small vessels that are potentially more likely to suffer the consequences of luminal occlusion due to CAV.(53) CFR has been used to predict the future development of CAV and cardiovascular events, suggesting that a CFR less than 2.5 is predictive for the presence of CAV. (51,54,55)This technique has also been used non-invasively using either doppler echocardiography or magnetic resonance imaging to measure changes in coronary flow in response to adenosine. These techniques have been validated with invasive testing and offer an opportunity for further research into their ability to predict the presence and progression of CAV. (56,57)

### 1.5.5 Non-invasive imaging

In children invasive imaging is a major undertaking, requires anaesthesia in the majority of cases, is expensive and has the potential for significant morbidity. These difficulties make non-invasive means of detecting CAV an attractive option. In general screening is undertaken on a yearly or biennial basis with coronary angiography. In our institution

those children older than 8 years are investigated with an early IVUS procedure 3 months and 1 year post transplant followed by biennial studies to assess progress.

Being able to detect the presence of CAV without invasive imaging is an attractive proposition and several non-invasive imaging techniques and molecular biology techniques have been tested. These include echocardiography, positron emmission tomography scanning, xray computerised tomography, magnetic resonance imaging, gene expression profiling and biomarkers. Despite this work, none has yet been shown to have sufficient sensitivity, and coronary angiography remains the most commonly utilized method for assessing CAV, in just the same way that endomyocardial biopsy is for rejection.(58)

### 1.5.6 Echocardiography

During routine follow up, particularly in children, repetitive invasive procedures are not practical and echocardiography continues to be the most frequently used modality for assessing cardiac status after transplant. There has been extensive research into the role that different echocardiographic parameters play in detecting rejection and CAV, particularly using diastolic dysfunction(59) and newer tissue tracking techniques providing information on myocardial mechanics rather than changes in volume and Doppler flow. However publication findings are rarely reproducible (60–62) and have not become routine in predicting either rejection or CAV. A completely normal echocardiogram with normal systolic and diastolic parameters has a strong negative predictive value for rejection and CAV though invasive testing particularly related to CAV is recommended.(63)

### 1.5.7 Dobutamine stress echocardiography

Dobutamine stress echocardiography (DSE) is a useful technique to screen for CAV. Exercise stress echocardiograpy is less useful because of cardiac de-nervation and a reduction in maximum heart rate. A specificity of 88% is reported when IVUS is used as the standard for presence of CAV. A normal DSE has a strong negative predictive value and recent guidelines recommend this non-invasive technique as a screening test prior to undergoing invasive angiography and IVUS.(64) Current evidence in children is limited and its routine use as a screening tool for CAV is limited to a small number of centres.(65,66)

### 1.5.8 Tomographic imaging (CT and MRI)

Experience using CT and cardiac MRI (CMR) to image coronary arteries is improving as the resolution and expertise of these techniques improves. They are limited by

inaccuracy related to cardiac motion and at the current time are unlikely to be able to reach spatial resolutions similar to those achieved with IVUS, particularly in paediatric heart transplant recipients with a fast heart rate; in this way, they may fail to detect subtle early changes.(67) As dual source CT scanners become more common the resolution is likely to improve and investigation into the value of CT in detecting CAV is increasing (personal experience). Radiation dosing is unlikely to exceed that seen in coronary angiography and with newer hardware is likely to be less. Under these circumstances screening with CT would seem a reasonable option. CMR is able to provide more than simply 2D imaging and is becoming more adept and detecting fibrosis and oedema. The ability to detect rejection using T2 relaxation time is useful for detecting rejection. (68) A further intriguing potential for MRI is to offer tissue enhancement with labelled gadolinium. Our group has already pioneered CMR sequences able to detect inflammation and correlate this with structural changes on IVUS. Labelling gadolinium with other biologically active molecules offers great promise for understanding not just structural changes but the biological mechanisms underpinning CAV(69)

### 1.5.9 Stress MRI

More recently techniques to measure myocardial perfusion are being developed using MRI techniques. This has been almost exclusively undertaken in the adult population after heart transplantation. Paediatric trials are being reported investigating myocardial perfusion with regadenoson, reversing with aminophyline and determining fibrosis with gadolinium. Differences in MRI structure and function have been identified, however this remains a research tool at the time of writing.

### 1.5.10 Biomarkers in the detection of CAV

Establishing the validity of a plasma biomarker able to detect those at risk of CAV, or one tracking the progression and the severity of coronary changes for an individual transplant recipient would be a significant step forward in monitoring post-transplant. In addition, identifying the biological processes involved in CAV aetiology through detection of effectors or products of these processes may provide valuable insight. The role of inflammation in the development of CAV has been previously described. (70) The presence of elevated serum vWF was a predictor for the development of CAV. (71) VEGF A and C and platelet factor-4 are strongly associated with CAV.(72,73) There has been significant research into the use of micro-RNA analysis as a biomarker for CAV development.(74,75) The role of anticoagulant pathways has also been explored providing insight into the pathological processes involved. (76–78) The CTOT-05

consortium, in a multi-centre analysis of immune biomarkers and outcomes, concluded that reliable biomarkers remain elusive. High levels of VEGF-C and endothelin-1 along with the presence of alloantibodies represent a high-risk group of recipients.(79) Increased levels of VEGF prior to the onset of detectable CAV by IVUS highlights its potential as an important biomarker.(80)

## 1.6 Prevention of CAV

### 1.6.1 Immunosuppression use

CAV is likely to be primarily an immune-mediated disease, and as a result preventative strategy is focused on protecting the donor heart from the recipient immune attack. The majority of drug protocols use calcineurin inhibitors (ciclosporin/tacrolimus) in combination with purine antagonists (mycophenolate mofetil/azathioprine). Less CAV is seen in patients receiving a combination of ciclosporin/MMF compared to ciclosporin/azathioprine.(81) The third main drug class is the proliferation signal inhibitors (PSI); everolimus and sirolimus. These drugs are similar agents, with everolimus having a shorter half-life. Encouraging results have been published, suggesting a reduction in intima-medial thickening and less CAV at a year post-transplant with PSI use, although in general, rejection rates are higher in protocols replacing purine antagonist with proliferation signal inhibitors.(82–85) Bearing in mind that CAV is worse in patients with increasing rejection these findings are intriguing. The mechanism of action creating this improvement is unknown, though it has been noted that PSI's have an anti-CMV action which may be beneficial. (86) A randomized control trial in children comparing everolimus/mycophenolate against tacrolimus/mycophenolate is in progress. (ClinicalTrials.gov Identifier: NCT03386539)

### 1.6.2 Statin use

From a non-immune perspective, HMG Co-A reductase inhibitors (statins) have shown to have a wide range of benefits after heart transplantation. Developed as a treatment for hypercholesterolemia, they can successfully have a lipid lowering effect on transplant patients who, as a result of a side effect of medication, have a tendency to increased blood lipids. A trial of pravastatin demonstrated a wide range of effects after transplant, including reduced serum lipid levels, less hemodynamic compromise during rejection episodes, reduced incidence of CAV and improved survival at 1 year.(87)Treatment of hypertension is beneficial after transplant and leads to less CAV,(88) and treatment with vitamin C and E as part of an antioxidant diet has been shown on IVUS to prevent the progression of CAV, presumably through reduction in oxidative stress. (89)

From a paediatric perspective the literature is divided. The use of Atorvastatin has been reported to be safe in children after heart transplantation,(90) though there is concern regarding its use in children under a year of age due to its effects on fat metabolism. As part of a multivariable analysis, the use of statins has been demonstrated to be protective against the development of CAV,(91) and when statins are started early after transplant it seems that they have a protective effect on the development of CAV.(92) However, in a larger registry study of 964 paediatric transplant recipients from the PHTS, no benefit was inferred from statin therapy in a multivariate analysis of CAV and overall survival.(93)

### 1.6.3 Induction therapy

Triggers for the development of CAV are likely to be present immediately after allograft reperfusion, and for this reason many centres use induction therapy. The agents used are primarily aimed at the adaptive immune response, targeting T- cells with ATG. More recently, monoclonal antibody to the CD25 receptor (Basiliximab) has been used with good results. Recent analysis from the ISHLT registry demonstrated a reduction in CAV associated with the use of induction therapy, although a definitive survival benefit has yet to be shown.(8) In adults treatment with ATG has been shown to limit the severity of CAV as detected by IVUS at 1 year.(85) Class II HLA-DR antigens are expressed on donor endothelial cells and are a key trigger for the immune response to the allograft. An increase in donor directed and non-donor directed HLA is associated with a poor outcome after transplant and is implicated in the development of CAV.(38,39,94)

### 1.6.4 Aspirin prophylaxis

The principal pathological mechanisms underlying the development of CAV are inflammation, platelet activation, thrombosis, smooth muscle proliferation and immune activation. Bearing this in mind, it is surprising that there is so little research into the use of aspirin for the prevention of CAV. A recent study has suggested that early aspirin treatment reduces the incidence of moderate to severe CAV in adults.(95,96) Aspirin is used in some centres as prophylaxis for CAV and further research in this regard should be encouraged.

### 1.6.5 Antihypertensives

Drawing from the benefit of medication in the use of atherosclerotic disease, the use of calcium channel blockers (CCB) and angiotensin converting enzyme inhibitors (ACEi) have been considered in the treatment of CAV. Limited evidence exists post-transplant, however some reports have demonstrated mixed results in the reduction of coronary

luminal narrowing with diltiazem and ACEi use.(97,98) Addition of these agents at diagnosis of CAV is not a well-established practice in children after heart transplantation. However prevention of hypertension is important after transplant and requires monitoring and treatment if present.

## 1.7 Management

### 1.7.1 Immunosuppression

It is also clear that changing patients to a PSI once CAV is detected on IVUS does not influence progression and that early treatment is required to gain the benefit offered by these newer agents.(99)

### 1.7.2 Anticoagulation

Once CAV is identified aspirin is frequently used to prevent ongoing thrombosis as well as prophylactically as described above.

### 1.7.3 Percutaneous interventional procedures

Experience with percutaneous coronary intervention for those children with stenosis is limited. Whilst there are often attempts at placing stents in older children with coronary stenosis outcomes are rarely altered.(100)

### 1.7.4 Retransplantation

Whilst retransplantation is an effective treatment for CAV only a small percentage of recipients receiving further cardiac transplants. The limited supply of organs, cumulative morbidity during the first transplant, and concerns regarding inferior outcomes contribute to the lack of consensus on attitudes towards retransplantation.(101)

## 1.8 Summary and comment on CAV

CAV remains a major limiting factor in the success of heart transplantation with an unclear etiology and a lack of effective treatment options. For children, CAV is severely life-limiting, as at the current time most children will survive to retransplant. Some key discoveries in the pathophysiological mechanisms involved have been made but as yet have been unable to consistently influence them on a clinical basis. Historically, CAV has been considered to be a form of “chronic rejection” and whilst immune factors are likely to be crucial to its development, we will need to focus on events early after transplant and develop novel drugs and protocols to manipulate the immune system so

that both the innate and acquired immune systems are appropriately suppressed. Harnessing methods by which the immune, inflammatory and coagulation systems can be naturally controlled is likely to be a critical component of preventing CAV in the future.

The research in this thesis takes these ideas forward with particular interest in investigating the role that inflammation and coagulation plays in the severity of CAV observed in a cohort of children following heart transplantation.

## 1.9 Thesis Aims

The initial aim of this thesis is to investigate whether there is evidence of systemic inflammation after heart transplantation in children by measuring biomarkers of inflammation and endothelial damage in the serum and comparing these results to healthy controls [Chapter 3].

Subsequently I will investigate whether the presence of systemic inflammation and endothelial damage is related to the presence of Cardiac Allograft Vasculopathy in the coronary circulation by invasively measuring the thickness of the coronary artery wall using intravascular ultrasound. [Chapter 4]

Finally this thesis will investigate whether the native, non-transplanted vasculature in the systemic circulation is affected by heart transplantation in children by measuring endothelial function in the brachial artery and the thickness of the carotid artery. In addition I aim to determine the interrelationships between the presence of native systemic vasculature abnormalities with coronary artery abnormalities in the transplanted heart. [Chapter 5]

## 1.10 Thesis Hypothesis

i. Allograft heart transplantation causes chronic inflammation and disorders of coagulation in the recipient when compared to healthy controls (**Error! Reference source not found.**)

ii. Chronic inflammation leads to endothelial damage and impaired endothelial function in both the coronary and systemic circulations, determined by serum biomarkers of endothelial damage.

iii. Endothelial dysfunction or damage predicts the development of arterial vasculopathy after cardiac transplant in children.

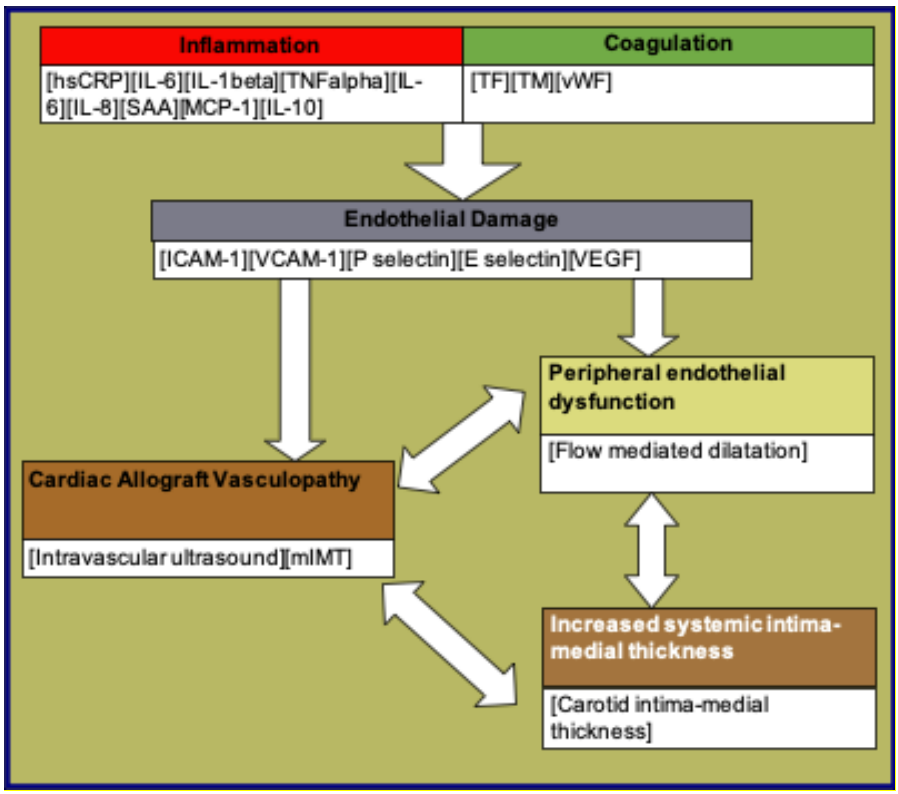


**Figure 1.10: Thesis hypothesis**

# 2 METHODS

## 2.1 Patients

Pediatric cardiac transplant recipients greater than eight years old were invited to take part in the study. Patients attended as part of their routine, post-transplant annual review for vascular studies. Children received a heart transplant for end-stage heart failure resulting from cardiomyopathy or congenital heart disease. For comparison, age and sex-matched controls were available from healthy siblings of participants enrolled into research projects within the department. Clinical data relating to age at transplant, time since transplant, current age, sex, donor age and previous biopsy evidence of rejection > grade 2R (or grade 3A) were collected. Details of medication usage was also recorded at the time of recruitment. Particular attention to immunosuppressant combinations, steroid and pravastatin use was included.

The ethical review board approved this study and patients/parents were consented prior to being enrolled. (Local REC 04CC17 – Institute of Child Health Ethics Committee).

From the perspective of the donor vasculature, coronary angiography and intravascular ultrasound (IVUS) procedures were performed, concurrently with arterial blood sampling for cytokine analysis, under general anaesthetic. The day before this procedure, peripheral endothelial studies were performed. Measurements of maximal flow-mediated dilatation (FMD) of the brachial artery as a marker of endothelial function, pulse wave velocity (PWV) and measurement of carotid intima-medial thickness (cIMT) to measure structural endothelial change were acquired.

## 2.2 Assessment of CAV with Intravascular Ultrasound (IVUS)

As part of this thesis I developed a technique to perform and reproducibly analyse IVUS images performed in children.

Under general anaesthetic a 5F sheath is placed using ultrasound access in the right or left femoral artery. Coronary angiography is performed of both the left and right coronary arteries using a 5F JR judkins catheter for the right coronary artery and a 5F JL judkins catheter, specially designed for IVUS imaging, for the left coronary artery. The ostium of the left coronary artery is intubated and contrast angiography is performed. Once luminal continuity of the left coronary artery is established using a contrast angiogram and stenosis of the coronary artery is confirmed to be absent, 150 mcg of GTN is injected into the coronary artery in order to prevent spasm of the coronary artery resulting from catheter manipulation. An 018” Balanced Middle Weight (BMW) intracoronary guidewire is then advanced into the left main stem and steered

under radiographic cine imaging into the left anterior descending coronary artery along the epicardial surface of the heart towards the apex. Careful monitoring of heart rate and ECG is continuous during this procedure.

A Boston Scientific 5F Opticross IVUS catheter is then advanced over the BMW guidewire. The tip of the catheter is placed in the distal left anterior descending coronary artery under radiographic imaging, without extending the IVUS imaging catheter beyond the tip of the coronary guidewire. An automated pullback is then performed, slowly withdrawing the IVUS imaging catheter at a rate of 0.5 mm/sec along the length of the coronary artery from distal to proximal.

IVUS images were acquired using either a Boston Scientific Galaxy 2 or iLab platform.The left anterior descending coronary artery was imaged to allow analysis of at least 15 cross-sectional images at intervals of every third cardiac cycle. Analysis of the cross-sectional images was performed, measuring vessel and lumen area, maximum and mean intima-media thickness (mIMT) and calculating maximum and mean percentage stenosis along the coronary artery. Stanford Grade was also determined for each study.(45) Mean IMT is calculated as the sum of the maximal thickness of each image divided by the number of cross-sectional images. Semi-automatic edge-detection software was used (QIVUS Clinical Edition, Medis Medical Imaging Systems, Leiden, Netherlands) to improve reproducibility.

Interobserver variability of this analysis technique was assessed by comparing in intra-class coefficient study as a measure of conformity between two experienced operators. Myself and Dr Michael Kuhn from Loma Linda University Hospital, California, USA. The intra-class correlation coefficient demonstrated excellent correlation between the two operators using the same analysis technique. (mean intimal medial thickness 0.91, maximum stenosis 0.79 and mean intimal index 0.91)

Prior to adopting this more reproducible technique Stanford Grade was used as the mechanism for reporting the severity of CAV. Stanford grade is measured by grading operator selected segments along the length of the coronary artery. The inherent selective bias associated with this technique raised concern regarding the reproducibility of this method, not just at a single time point but also in the context of being able to measure identical segments of the coronary artery at different points of time during follow up.

The current method was initially developed by me using ImageJ® software to manually trace digitally acquired, cross sectional images of the coronary artery, measuring the lumen and EEM borders. This proved to be extremely labour intensive and did not

represent a realistic method for detailed analysis of IVUS images into the future. I adapted the Medis Imaging QIVUS software, used for measuring stents in coronary arteries, to provide semi-automatic edge detection. This provided a more reproducible robust technique and has been published in peer reviewed journals subsequently.(102) This technique has also been used to compare cohorts of patients followed up in different centres with excellent interclass correlation coefficients, highlighting this technique as a robust method for analysing IVUS images for research and between different centres.(103)

The safety of this technique has also been determined in an international collaborative paper using this technique with very few significant complications, given the perceived invasiveness of the procedure.

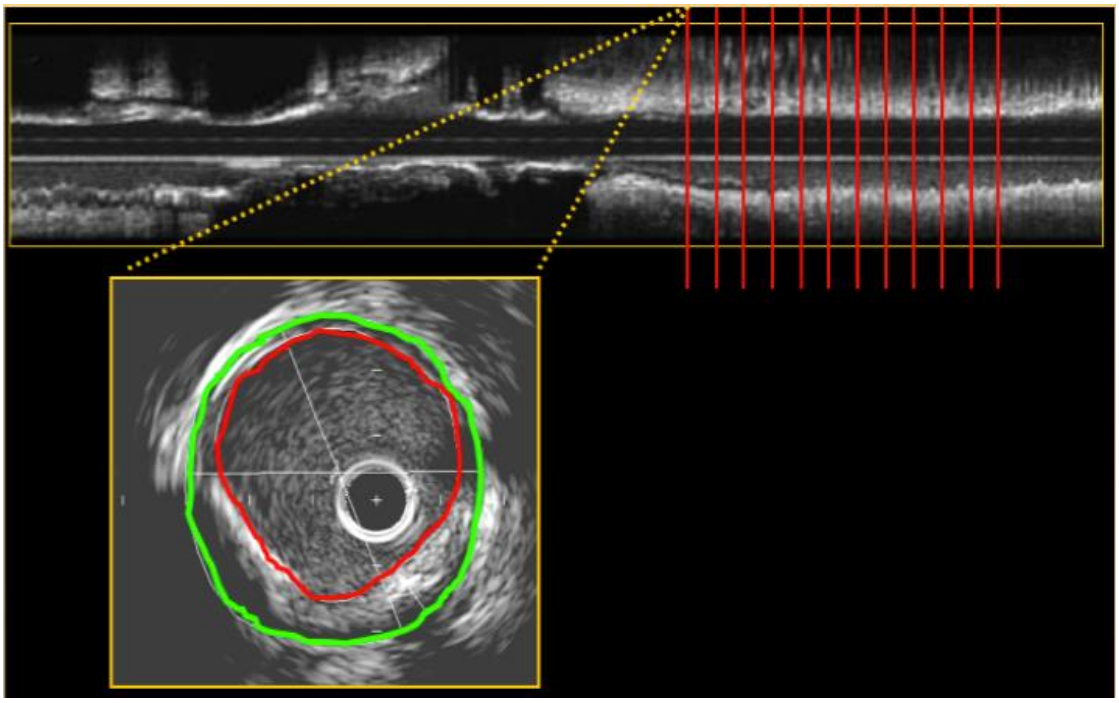

**Figure 2.1 Method of IVUS analysis. An automated pullback is performed along the length of the LAD. Each image selected every third cardiac cycle is analysed. The luminal border (red) and EEM border (green) are traced using edge detection algorithms. The minimum and maximum diameter are then calculated for the lumen and EEM respectively.**

Mean IMT was used as the primary outcome marker for CAV assessment. This parameter was chosen as it reflects the degree of intimal hyperplasia along the entire length of the coronary artery and not just at the point of the most severe disease as Stanford Grade does. Patients with mIMT > 0.5 mm were defined as having severe CAV. Previous studies have identified this IMT value as predictive of future cardiovascular events.(47,104,105)

## 2.3 Assessment of inflammatory indices

Circulating soluble markers of systemic and vascular inflammation were studied using a multi- parametric approach to explore potential relevant inflammatory pathways. High sensitivity C reactive protein (hs-CRP), serum amyloid A (SAA), TNF-α, interleukin (IL) 1β, 6, 8 and 10, monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble P selectin, soluble E selectin, TM and vWF were assessed using a multi-array detection system based on electro-chemiluminescence technology (SECTOR Imager 2400, MesoScale Discovery).

This system uses multi-array plates fitted with multi-electrodes per well with each electrode being coated with a different catching antibody. The assay procedure then follows that of a classic sandwich ELISA with the analytes of interest captured on the relevant electrode. These captured analytes were then in turn detected by a secondary analyte-specific ruthenium-conjugated antibody, which is capable of emitting light after electrochemical stimulation. A particular advantage of this system is the ability to simultaneously measure different biomarkers in small (25ul or 50uL) serum or plasma samples. TF was measured by sandwich enzyme immunoassay using a commercially available kit from R & D Systems, Europe Ltd, (Abingdon, UK). Samples were measured using paired samples to ensure accuracy.

Sandwich ELISA is a highly sensitive technique for detecting the antigen to be measured. A capture antibody is coated to the detection plate and serum is added with the antigen present. The antigen then binds to an epitope of the antibody coated to the surface of the plate. A detection antibody is then added, the antigen binds to a different epitope of the detection antibody. The detection antibody is then able to emit light through a redox reaction using a ruthenium-conjugated antibody

Samples for analysis were collected during cardiac catheterisation procedures by me, centrifuged and stored in a -80 degree freezer facility for batch analysis. I am grateful to Vanita Shah for special expertise measuring the antibodies with the SECTOR imaging system.

## 2.4 Markers of native artery vascular stiffness, endothelial dysfunction and carotid intima media thickness

Ultrasound procedures were undertaken by trained colleagues due to the need for blinding in the study. (Libby Ellins and Marietta Charakida). I was present at scans to record data and measurements and to ensure blinding to other clinical data. Analysis

of all data in this thesis was performed by me.

### 2.4.1 Flow mediated dilatation

The right brachial artery was imaged with high-resolution ultrasound (Prosound SSD-5500, ALOKA, Tokyo, Japan). Inflating a blood pressure cuff to 200 mmHg for 5 minutes induced forearm ischemia; reactive hyperemia followed cuff deflation. Changes in brachial artery diameter were measured offline with an automated edge detection system (Brachial Tools, Medical Imaging Applications, Coralville, Iowa) and calculated as a percentage change from baseline diameter. Blood flow was measured continuously with a pulsed-wave Doppler signal. Maximal increase in blood flow was expressed as a percentage change from baseline flow. Blood pressure was initially taken after resting for 5 minutes and after FMD with an OMRON M5-I sphygmomanometer (OMRON, Kyoto, Japan). Trained operators who were blinded to all clinical history of the patients performed all studies in a temperature-controlled vascular laboratory.

### 2.4.2 Pulse Wave velocity (PWV)

The technique most widely used to measure arterial stiffness is the determination of arterial PWV. PWV is the speed of travel of the pulse along an arterial segment. PWV was measured using the SphygmoCor system (SphygmoCor version 7.0, Millar Instruments, ScanMed Medical, Gloucestershire, UK). The pressure waveform was recorded consecutively in the carotid and radial arteries with an electrocardiogram signal that provides an R-wave timing reference. PWV was then calculated using the mean time difference in the carotid and femoral arterial length. Path length was defined as the distance from the suprasternal notch to the radial pulse. PWV is defined by the time difference between carotid and radial peak pulsation divided by the distance. Observers blinded to the clinical condition of the child performed all measures.

### 2.4.3 Carotid intima-medial thickness measurement (cIMT)

Measurement of far wall cIMT with ultrasound is a non- invasive and reproducible technique for identifying and quantifying vascular disease and for evaluating cardiovascular risk. For cIMT measurement, B-mode ultrasonography of both common carotid arteries was performed using a 12-MHz linear array transducer (Vivid 7; GE Medical, Horton, Norway). Longitudinal two-dimensional images of the vessel 1–2 cm proximal to the carotid bulb were acquired on the R-wave of the electrocardiogram. Images were focussed on the posterior (far) wall of the artery and the zoom function was used to magnify the area. Ten second cineloops were recorded in DICOM format

and downloaded for offline analysis. Three end-diastolic frames were selected and analysed for mean cIMT, defined as the interface between lumen-intima and media-adventitia, for both right and left carotid arteries using an automated carotid analyser (Carotid Analyser, M.I.A). The cIMT was calculated as the distance between the leading edge of the lumen-intima interface and the media-adventitia interface on the far wall of the artery. The images were analysed by accredited readers and the mean of both the left and right-sided readings was used for the analysis.

## 2.5 Statistical Analysis of results

All data were analyzed using SPSS (version 21.0; SPSS, Inc., Chicago, IL) or Graphpad Prism. Continuous data were expressed as mean +/-SD (if normally distributed) or median (IQR) (if not normally distributed). Between group comparisons were made using unpaired or paired Student's t-test or Mann–Whitney U-test as appropriate to the underlying distribution of the variables. Linear regression analysis was used to investigate the relationship between cytokine levels and mean intima-medial thickness. Patients post-transplant were divided into two groups based on mIMT measurements. Those patients with mIMT > 0.5 mm were considered to have severe CAV with a comparison of data for each group.

# 3 IS THERE EVIDENCE OF INFLAMMATION AFTER TRANSPLANT?

## 3.1 Introduction

The purpose of this results section is to establish a relationship between inflammation, endothelial activation and heart transplantation. As discussed as part of the methods a panel of biomarkers was used to explore differences between patients after heart transplantation compared with healthy controls.

The process of identifying differences in the serum profile between these two groups will help to delineate the pathophysiologic processes involved by drawing on established knowledge of the involvement of individual proteins in disease.

This being a clinical cohort with differences in clinical details both before and after transplant it is important to consider the multiple confounding factors that might influence the levels of the various measurements To this end multivariate analysis has been performed as well as investigating the individual contribution each of this differences might play in the profile of inflammation and endothelial activation obtained.

## 3.2 Aim

- To determine whether transplantation causes inflammation and activation of endothelial adhesion molecules
- To determine if activation is related to any demographic parameters before or after transplant.
- To determine whether the use of medication after transplant has any influence on cytokine levels that might influence our interpretation of results.

## 3.3 Results

### 3.3.1 Patient demographics

48 consecutive children (25 male) aged 8 to 18 years were enrolled into the study at the time of their routine annual review. These 48 patients provide the dataset throughout this thesis in this chapter and in chapters four and five. Patients were median (IQR) 4.1 (2.2 to 8.7) years after cardiac transplantation. Mean (SD) donor age was 18.6 (13.7) years. Previous rejection episodes occurred in 14 (29%) patients with no evidence of clinical rejection in patients at the time of the study. Routine biopsies were not performed at the time of the study. (Table 3)

**Table 3. Patient demographics**

| | Median | Variance |
|---|---|---|
| Female sex | 23 | |
| Age at study (years) | 14.1 | 12.0 - 15.9 |
| Time post transplant (years) | 4.1 | 2.2 - 8.7 |
| Age at transplant (years) | 9.5 | 5.1 - 13.2 |
| Ischaemic time (minutes) | 196 | 141 - 217 |
| Rejection episodes | 14 (29%) | |
| Donor Age (years) | 17 | 9 - 29 |

### 3.3.2 Post-transplant management

The majority of patients (36/48) were treated with a combination of tacrolimus/mycophenolate (17/48) or tacrolimus/azathioprine (19/48). Three patients received tacrolimus monotherapy and ciclosporin monotherapy in one. Of the remaining, 7 were treated with tacrolimus, 1 in combination with mycophenolate and sirolimus, 2 with mycophenolate and steroids, 2 with sirolimus and steroids and 2 with steroids alone. The remaining two patients were treated calcineurin inhibitor free with one receiving mycophenolate and sirolimus and the other the same with steroids. Overall 7 (15%) patients remained on steroids and 5 (10%) patients were receiving sirolimus. Pravastatin was used in 43 (90%) patients.

### 3.3.3 Comparison of cytokines and adhesion molecules with controls

A comparison of serum cytokine and adhesion molecule levels between patients and controls is presented in Table 1 with graphical depiction of significant differences in Fig.1. No significant difference was detected between the age and sex distribution of the patients and controls.

Serum levels of IL6, VCAM1, ICAM1 and TM were all greater than controls. Serum levels of E selectin, P selectin, and TNF alpha were significantly lower in patients when compared with controls. (**Error! Reference source not found.**). Scatter plots showing those cytokines demonstrating significant differences between patients and controls

can be seen in Figure 3.1. Scatter plots showing cytokines where there is no significant difference between patients and controls is shown in Figure 3.2.

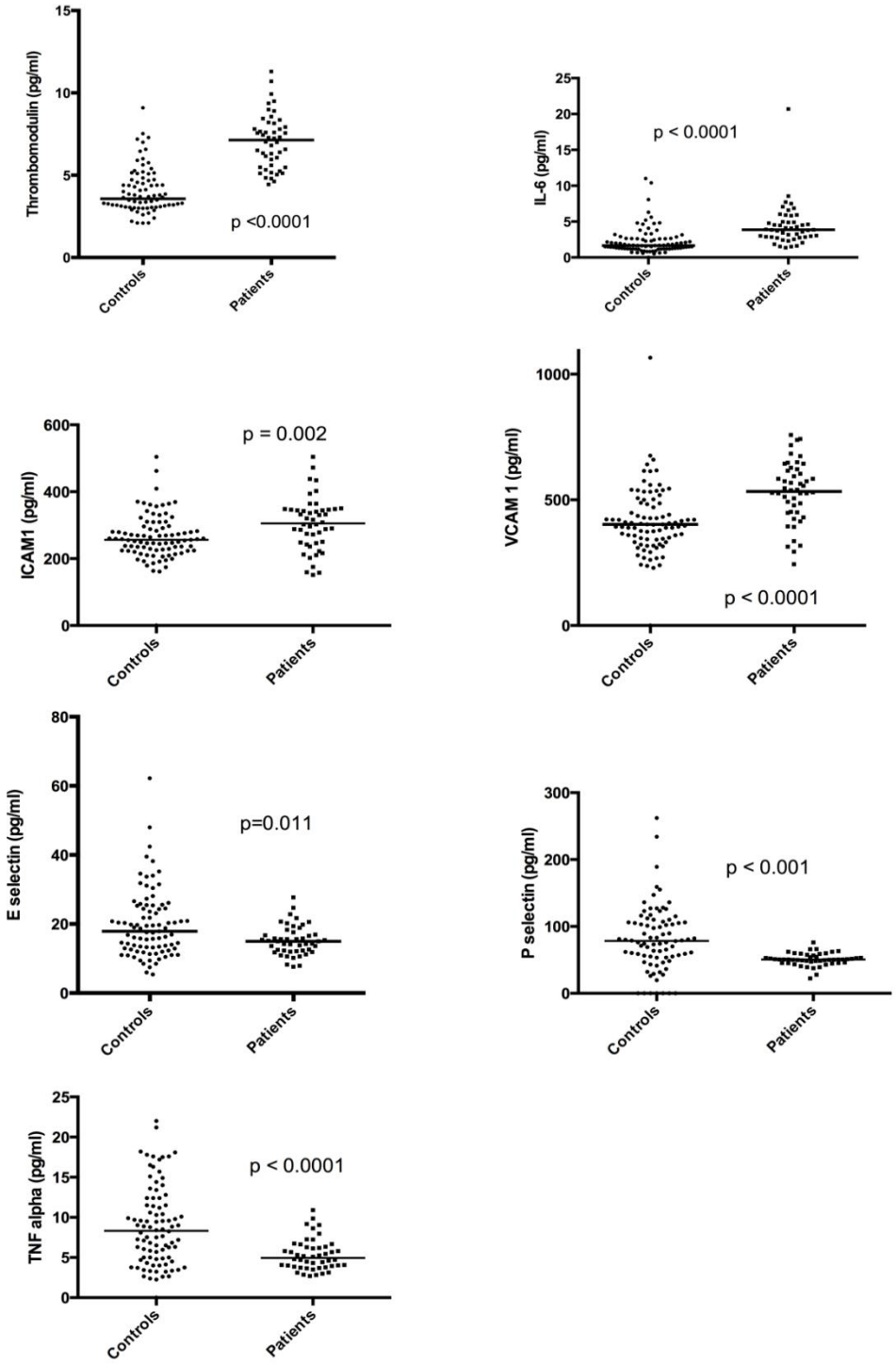


**Figure 3.1. Scatter plots demonstrating <u>significant</u> differences between patients and controls for levels. Values are expressed in pg/ml.**

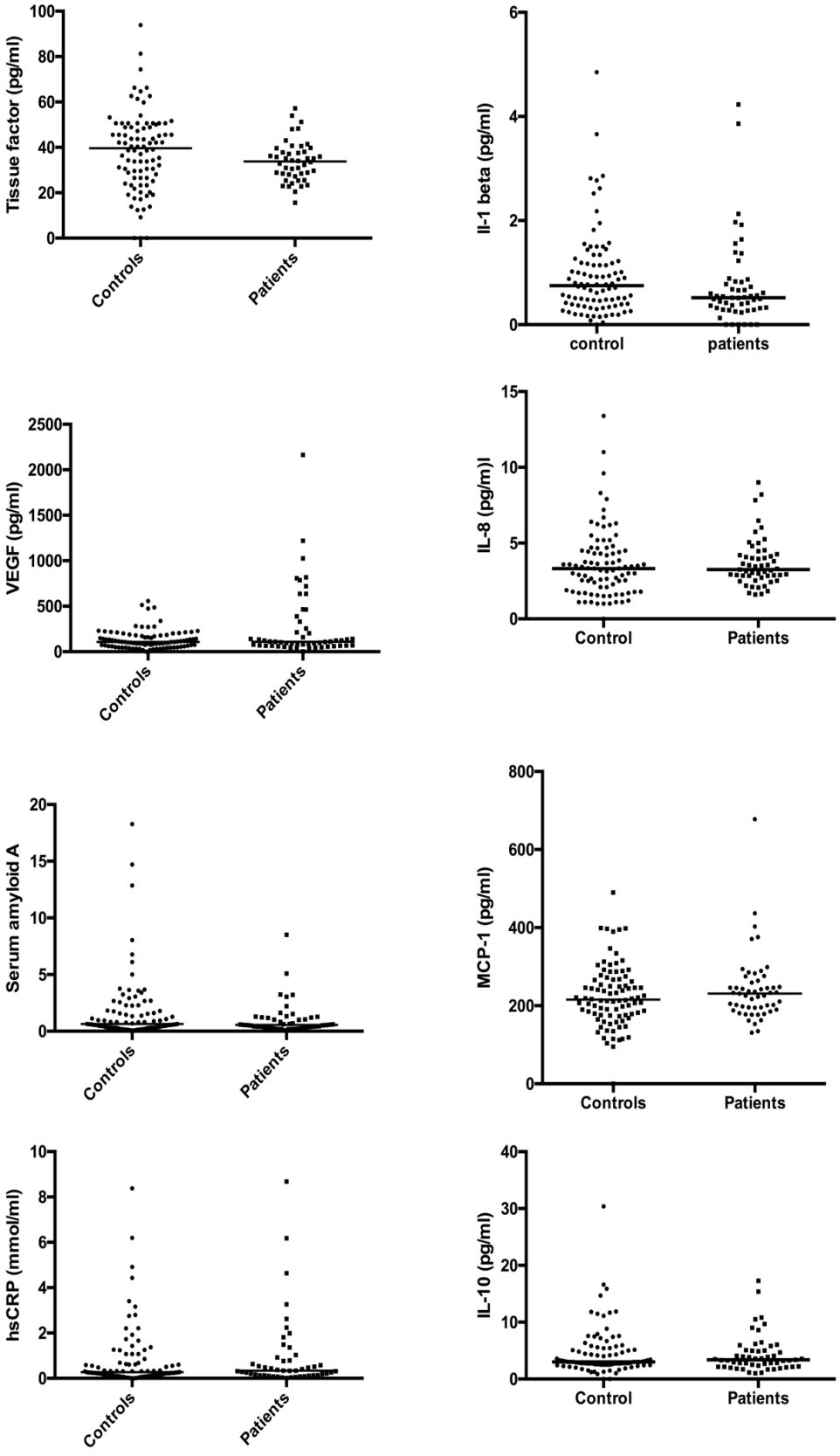


**Figure 3.2. Scatter plots demonstrating <u>non-significant</u> differences between patients and controls for serum cytokine levels. Values are expressed in pg/ml.**

**Table 4 Serum cytokine levels of study participants compared to controls**

| | Patients | | Controls | | |
|---|---|---|---|---|---|
| | N | Value | N | Value | p |
| IL 6 | 48 | 3.86 (2.84-4.95) | 90 | 1.66 (1.22-2.63) | <0.0001* |
| ICAM 1 | 48 | 305 (247-346) | 89 | 256 (224-294) | 0.002* |
| VCAM 1 | 48 | 539 (451-621) | 89 | 402 (342-487) | <0.001* |
| TM | 48 | 7.1 (5.5-8.1) | 84 | 3.57 (3.03-4.71) | <0.0001* |
| TNFα | 48 | 4.96 (3.89-6.34) | 90 | 8.33 (4.87-11.73) | <0.0001* |
| E selectin | 48 | 15 (12.2-17.4) | 89 | 17.9 (12.8-24.9) | 0.011* |
| P selectin | 48 | 50.5 (45.6-56.1) | 84 | 78.4 (53.9-106.8) | <0.001* |
| Tissue Factor | 41 | 33.6(27.6-38.8) | 90 | 39.7 (26.5-49.2) | 0.052 |
| IL 1β | 48 | 0.55 (0.30-0.86) | 90 | 0.75 (0.39-1.18) | 0.121 |
| VEGF | 48 | 116 (67-373) | 90 | 107 (61-179) | 0.121 |
| IL 8 | 48 | 3.39 (2.86-4.26) | 90 | 3.33 (1.88-4.5) | 0.33 |
| SAA | 48 | 0.57 (0.37-1.22) | 89 | 0.65 (0.33-2.04) | 0.34 |
| MCP 1 | 48 | 228 (191-260) | 90 | 215.5 (176-267) | 0.44 |
| hsCRP | 48 | 0.34 (0.13-0.89) | 89 | 0.27 (0.14-0.77) | 0.48 |
| IL 10 | 48 | 3.43 (2.16-5.63) | 90 | 3 (2.5-5.5) | 0.86 |

### 3.3.4 The influence of patient demographics on cytokine and adhesion molecule levels

Linear regression analysis was performed to determine cytokine levels in relation to age at transplant, time since transplant, current age and donor age.

#### 3.3.4.1 Cytokine results and age at transplant

Multivariate regression analysis of serum levels against age at transplant did not produce a statistically significant model. There does not seem to be any meaningful relationship between age at transplant and cytokine or cell adhesion molecule serum measurements. (Table 5) $F(16, 22) = 1.36$, adj $R^2 = 0.131$, $p = 0.248$.

**Table 5. Regression result for cytokines against age at transplant**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | Durbin-Watson |
|---|---|---|---|---|---|
| Age at Transplant | .705[a] | 0.497 | 0.131 | 4.20598 | 0.852 |

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Age at Transplant | Regression | 384.825 | 16 | 24.052 | 1.36 | .248[a] |
| | Residual | 389.185 | 22 | 17.69 | | |
| | Total | 774.01 | 38 | | | |

a. Predictors: (Constant), Thrombomod, IL8, IL10, IL1B, vWF, P_SELECTIN, VEGF, sE_SELECTIN, sVCAM_1, Tissue_Factor, IL6, hsCRP, TNFALPHA, sICAM1, MCP1, SAA

#### 3.3.4.2 Cytokine results and time from transplant

Multivariable regression analysis of serum levels against time from transplant did not produce meaningful correlation nor produce a statistically significant model. There is no statistical relationship between time post-transplant and cytokine or cell adhesion molecule serum concentrations.(Table 6Table 6. ) $F(16, 22) = 0.862$, adj. $R^2 = -0.062$, $p=0.614$.

**Table 6. Regression results for cytokines against time form transplant**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | Durbin-Watson |
|---|---|---|---|---|---|
| Time from transplant | .621[a] | 0.385 | -0.062 | 3.64038 | 0.715 |

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Time from transplant | Regression | 182.816 | 16 | 11.426 | 0.862 | .614 |
| | Residual | 291.552 | 22 | 13.252 | | |

| | | | | | |
|---|---|---|---|---|---|
| | Total | 474.369 | 38 | | |

a. Predictors: (Constant), Thrombomod, IL8, IL10, IL1B, vWF, P_SELECTIN, VEGF, sE_SELECTIN, sVCAM_1, Tissue_Factor, IL6, hsCRP, TNFALPHA, sICAM1, MCP1, SAA

### 3.3.4.3 Serum levels and current age

Multivariable regression analysis of serum levels against current age did not produce meaningful correlation nor produce a statistically significant model. There is no statistical relationship between time post transplant and cytokine or cell adhesion molecule serum measurements on a general level. (Table 7) $F(16, 22) = 1.89$, adj. $R^2 = 0.00$, p=0.489.

For individual cytokines a relationship between increasing P selectin levels and decreasing age was observed with a standardised coefficient (β), -1.6, p=0.013*. No other individual observations were observed.

**Table 7. Regression analysis for results of cytokines against current age**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | Durbin-Watson |
|---|---|---|---|---|---|
| Current age | .649 | 0.421 | 0.00 | 2.28 | 1.991 |

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Current age | Regression | 83.6 | 16 | 5.224 | 1.0 | .489[a] |
| | Residual | 114.8 | 22 | 5.219 | | |
| | Total | 198.4 | 38 | | | |

a. Predictors: (Constant), Thrombomod, IL8, IL10, IL1B, vWF, P_SELECTIN, VEGF, sE_SELECTIN, sVCAM_1, Tissue_Factor, IL6, hsCRP, TNFALPHA, sICAM1, MCP1, SAA

### 3.3.4.4 Serum levels and donor age

Multivariate linear regression analysis was performed to investigate the impact of donor age on levels of cytokines and cell adhesion markers. Initial analysis with all biomarkers entered into the model on an equal basis demonstrated a model with borderline significance. Serum biomarker levels failed to predict donor age, $F(4, 95) = 1.89$, adj. $R^2 = .58$, p=0.082.

A number of cytokines reached statistical significance with respect to their individual contribution to the model. A stepwise analysis was performed with individual variables included that reached a signficance level of $p < 0.05$. The final model predicted donor age in a statistically significant manner, F(3,35) = 8.75, adj. $R^2 = 0.38$, $p = < 0.001$*.

Standardised and unstandardised coefficients for the model are detailed in Table 8below.

**Table 8. Regression coefficients for serum levels vs donor age**

| Variable | B | $SE_B$ | β | P value |
|---|---|---|---|---|
| Intercept | 50.2 | 12.3 | | .000* |
| vWF | 20.4 | 5.6 | 0.47 | 0.001* |
| TM | -3.02 | 0.20 | -0.28 | 0.003* |
| P selectin | -0.43 | 0.198 | -0.28 | 0.037* |
| vWF, von Willebrand factor, B unstandardised coefficient, $SE_B$, standard error B, β , standardised coefficient | | | | |

The model demonstrates that increasing serum levels of vWF are associated with increasing donor age and that decreasing levels of TM and P selectin are associated with increasing donor age.

### 3.3.5 The influence of previous rejection on cytokine and adhesion molecule levels

Whilst there was no patient with clinical rejection at the time of inclusion into the study, a number of patients had rejection earlier in post-transplant. Rejection was defined as ISHLT grade > 3A in previous classification or > 2R in recent morphology descriptions (5). This is because the classification was changed during the collection of the data for this study.

There was no statistical significance to the overall model and no contribution of any individual cytokine or adhesion molecule to independently predict the presence of prior rejection. $F(16, 22) = 1.01$, adj. $R^2 = 0.04$, p=0.482. Independently patients with previous rejection episodes had higher ICAM 1 (β = 0.39, p = 0.02), VCAM 1 (β = 0.34, p = 0.04) and MCP1 (β = 0.35, p = 0.03) levels. (Table 9)

**Table 9. Regression analysis results for the impact of previous rejection on cytokine levels.**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | Durbin-Watson |
|---|---|---|---|---|---|
| Previous rejection | .651 | .424 | .004 | .454 | .816 |

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Previous rejection | Regression | 3.35 | 16 | .209 | 1.01 | .482 |
| | Residual | 4.553 | 22 | .207 | | |
| | Total | 7.90 | 38 | | | |

a. Predictors: (Constant), Thrombomod, IL8, IL10, IL1B, vWF, P_SELECTIN, VEGF, sE_SELECTIN, sVCAM_1, Tissue_Factor, IL6, hsCRP, TNFALPHA, sICAM1, MCP1, SAA

### 3.3.6 The influence of medication on cytokine and adhesion molecule levels

The effect that different drugs have on both cytokine and adhesion molecule serum levels was investigated in the post transplant recipients. When dichotomised into groups that were either taking the medication or not, TM levels were lower in those patients receiving pravastatin (6.8(5.4-7.8) vs 8.4(7.6-8.9); p 0.037) (n=43, 90%) and in patients treated with sirolimus (5.4(4.8-6.8) vs 7.2(5.96-8.2) p 0.48) (n=5, 10%). Patients taking steroids have lower IL10 (2.14(1.83-2.83) vs 3.6(2.4-6.0)) and IL6 (3.0(2.5-3.18) vs 4.1(2.9-4.2) serum levels (n=7, 15%).

## 3.4 Discussion

### 3.4.1 Significant differences in blood markers between patients and controls

These data suggests that significant differences exist in the serum profile of selected inflammatory cytokines, cell adhesion molecules, and coagulation proteins in clinically well, paediatric heart transplant recipients when compared to healthy children.

Our data demonstrates that serum levels of IL 6 were elevated after heart transplantation, suggesting the presence of subclinical inflammation. IL 6 is well known as a pro-inflammatory cytokine, secreted by T cells, macrophages, and endothelial cells in response to infection and tissue damage. After heart transplantation, elevated levels of IL 6 in the coronary sinus have been reported as being predictive for rejection episodes and correlate with abnormalities in coronary endothelial function(106,107). Although none of the patients in this study were experiencing acute rejection, we did correlate increased levels of both IL 6 and MCP 1 in patients with previous rejection episodes, suggesting that subclinical rejection might be present.

Levels of other pro-inflammatory cytokines were not found to be elevated and this warrants comment. Elevated levels of IL1 beta, IL8, TNF alpha, MCP 1, hsCRP and SAA are known to be involved in the inflammatory response but were not significantly elevated in our patients. In fact TNF alpha was found to have lower levels. TNF alpha is a cytokine known for its role as a driver of inflammation and the acute phase response. TNF alpha levels are increased during acute rejection, and are reported to be increased early post graft implantation.(108,109) The lower levels in our patients are therefore seemingly at odds to this. However nearly all of the patients included in our study were treated with statins and were not acutely rejecting their grafts. It is possible that the anti-inflammatory properties of pravasatin might account for the lower TNF alpha levels and perhaps explains the absence of a rise in other proinflammatory cytokines, other than IL6. Studies investigating the effects of statin treatment on inflammation demonstrated similar results with a fall in TNF alpha and P selectin, without a fall in ICAM1 and VCAM1 levels.(110)

CRP is a predictor for the development of CAV after transplant, is associated with endothelial activation,increased ICAM1 levels and angiographic evidence of CAV.(111–113) Surprisingly CRP was not elevated in our patients and did not predict the development of severe CAV. Treatment with pravastatin has been shown to reduce CRP levels in transplant patients and this may explain our contradictory result.(112)

Given the evidence for subclinical inflammation (increased IL 6 levels), it is important to consider whether endothelial activation is present in our patients, as demonstrated by alterations in serum levels of endothelial adhesion molecules. These proteins—bound to the endothelial surface—are involved in the contact-dependent infiltration of leukocytes initially with and then through the endothelial cells and into the media and adventitia of the cardiac allograft. In our patients, serum levels of ICAM1 and VCAM1 were elevated and E and P selectin levels were reduced. Increased ICAM1 expression in biopsy specimens early after transplant as well as increased serum levels are predictive for CAV development(114,115) and VCAM1 is associated with T cell recruitment at sites of alloimmune and non-alloimmune inflammation.(116)The role of VCAM1 in the development of CAV and as a result endothelial activation is supported by findings in adult heart transplant recipients.(117,118) In contrast, levels of E selectin and P selectin might also be expected to be raised, but our study had lower serum levels. E selectin and P selectin are cell adhesion molecules expressed on the endothelium and platelets; they play an important role in initial leukocyte adhesion to the endothelium. Expression of these adhesion molecules is stimulated in part by TNF-α. Due to our observation that TNF-α levels were low in our patients, presumably due

to statin therapy, E selectin and P selectin might also be lower as a result of the immunosuppressive regimen and/or statin therapy(110)

### 3.4.2 Influence of medication on cytokine levels

The observational nature of this study has meant that the participants are treated with immunosuppressant and adjunctive medication that was not predefined as part of a trial protocol. This results in variations in treatment that have arisen from clinical decisions made during follow up. In general the patients were treated with broadly similar groups of medication. Only three patients were treated with a combination of drugs that did not involve Tacrolimus and only two patients were free of calcineurin inhibitors. However, it is important to consider the role that differences in medication might have in altering the inflammatory profile, particularly as many of these drugs are anti-inflammatory in nature and are known to have wide-ranging and often poorly described mechanisms of action, causing an unanticipated effect on our analysis.

The number of patients receiving adjunctive immunosuppression in this cohort was small with only a handful of patients receiving steroids or sirolimus. The small numbers make interpretation of results difficult as we cannot be clear how differences in cytokine and cell adhesion molecule levels are influenced by the combinations of medication. However, we did observe that when divided into comparative groups, levels of TM were lower in patients receiving sirolimus and/or pravastatin. As discussed in the introduction, TM is an important endothelial protein with biological actions primarily related to the control of natural anticoagulation but may also play a significant role in diverse biological pathways including inflammation.

Statin treatment has previously been shown to increase TM mRNA levels,(119) which intuitively would be more consistent with its biological mechanisms as a moderator of the immune response and known anti-inflammatory effect.(120) Our results appear contradictory to this but the small numbers make our data unreliable and it is inadvisable to draw any firm conclusions based on these observations. In our data, TM levels are also noted to be lower in patients who are being treated with sirolimus. This medication is used where worsening CAV is detected as part of routine follow up. Studies in atherosclerosis have identified that TM may be increased in patients at risk of disease and low in patients once coronary artery disease is established. As we will see in the next chapter low levels of TM are related to the presence of established CAV and as a result the presence of sirolimus may be related to this.(121)

## 3.5 Conclusions

In this chapter we have compared serum levels of inflammatory cytokines and markers of endothelial adhesion for lymphocytes and platelets. We have measured serum levels of TM, part of the natural anticoagulation system, that increases the activity of activated protein C (APC) and attenuates the inflammatory response.

We have observed that levels of IL 6 are elevated in children after heart transplantation when compared to controls, suggesting that non-clinical inflammation is present. Levels of TNF alpha would have been expected to be high but may have been attenuated by the presence of statin therapy in the majority of participants, for which there is supporting evidence.

We have observed that serum levels of vascular adhesion molecules for platelets and leucocytes are elevated in the serum. We might conclude from this that either there is increased activity at endothelial level or that damage to the endothelium has displaced these proteins into the serum. Similarly, serum levels of TM are also raised in children after transplant which, based on its important role in regulating endothelial function, is an interesting finding when considering the role of the endothelium in preventing CAV after heart transplantation.

In the next chapter we will relate these findings to the presence of CAV in our cohort of patients and discuss further the role of inflammation and endothelial function on the development of CAV.

# 4 INFLAMMATION AND SEVERITY OF CARDIAC ALLOGRAFT VASCULOPATHY

## 4.1 Introduction

Understanding CAV and its aetiology in the paediatric population is an important undertaking. Transplant outcomes demonstrate acceptable progress when perioperative success is considered but have lacked significant improvement in medium to long term mortality, partly attributable to a lack of understanding and treatment options for CAV. Clinical protocols have been broadly extrapolated from adult studies and fail to take into account differences between the adult and paediatric populations. The number of children benefiting from heart transplantation is relatively few and as a result without collaborative studies we lack the power to reach statistical significance, except for when the standard deviation of our outcome measures is small.

Using IVUS provides us with an opportunity to research CAV in children because of the sensitivity of the method I have developed, providing an accurate and reproducible assessment of the coronary vasculature. This robust and reproducible outcome marker can be used to provide insight into the biological changes that distinguishes those patients with significant disease versus those without. The technique for analysis documented in this thesis was developed by me to provide a thorough assessment of the coronary vasculature, incorporating detailed analysis of coronary artery geometery as part of this technique. Utilising the whole length of the coronary artery, incorporating a systematic approach to include images at regular points along the coronary artery, removes selection bias and provides a robust method for comparison between studies. Improvement in software providing edge detection algorithms for ultrasound imaging has streamlined the approach and enabled detailed analysis to be performed within a reasonable timeframe that is appropriate and feasible for use in clinical research. The technique detailed in the methods section was developed by me to enable this research within the framework of this thesis. The reproducibility and validity of this technique between two transplant centres, Great Ormond Street Hospital, London and Loma Linda Childrens Hospital, USA is available for review as a published peer reviewed paper in the appendix.

The indication for heart transplantation in children is different to adults. Whilst the majority of both adult and paediatric patients require a heart transplant for cardiomyopathy, a large proportion of adults have developed end stage heart failure as a result of ischaemic heart disease (IHD) and are affected by additional risk factors that accompany this diagnosis. One study determining the extent of inflammation post transplant had a pretransplant IHD diagnosis in 43% of patients. (118) In particular it is likely that they will have co-morbid conditions including hypertension, diabetes and lipid metabolism disorders. These factors are consistent with non-immune risk factors for

CAV and are carried forward into heart transplantation by nature of the recipients' age and indication for heart transplantation. In contrast paediatric heart transplant recipients are unlikely to have developed these comorbidities, particularly as a proportion of the recipients will have been transplanted as a result of congenital cardiac abnormalities not amenable to further corrective surgery and at an age where comorbidities have not developed.

I have discussed earlier in the introduction the biological processes involved in the development and progression of CAV. The endothelium plays a crucial role early in CAV. Researching how the endothelium is affected by heart transplantation is a crucial part of understanding the development of CAV. Determining the pathogeneis of CAV is crucial to identify targets for future treatment. The endothelium is involved in multiple pathways and orchestrates biological processes involved in immune activation by inflammatory cells, adhesion of leucocytes to the endothelium and subsequent migration into the tissue and reparation of damage through the coagulation cascade.(122) I am aware from extensive work over the last two decades the damaging role that inflammation plays in the development of non-transplant atherosclerosis and the association between diabetes, autoimmunity and chronic infections.(123,124)

Studying CAV in children provides an opportunity to examine the inflammatory response after transplant without the additional co-morbid factors, assess changes in serum levels of endothelial activation and measure components involved in the control of coagulation.

### 4.1.1 Markers of inflammation post-transplant

The histological presence of inflammatory cells localised at the endothelium indicates that CAV is an inflammatory process. Non-transplant atherosclerosis has been considered an inflammatory process for some time and evidence has been gathered identifying the presence of inflammatory cytokines in patients post-transplant. The immediate response post implantation of the allograft leads to cytokine release, including IFN$\gamma$ and a chain of events leading to IL1, IL6, TNF$\alpha$ to name only a few. A seminal paper from 1998 investigated the presence of inflammatory markers in the biopsy specimens of patients with and without histological features of rejection.(22) This demonstrated an increase in inflammatory cytokines (IL1 beta, TNF alpha), increase in adhesion molecules (ICAM1 and vWF) and reduction in natural anti-coagulant pathways (TM and antithrombin III) during rejection episodes. This paper highlights the importance of rejection episodes in providing the impetus for the development of CAV. We know that an increased incidence of acute cellular rejection

(ACR) leads to a higher incidence of CAV and that ACR in the first year leads to worse long-term survival.(8,125) ACR more than two years after transplantation is unusual, however CAV continues to develop even in the absence of rejection episodes, implying that either early immune events trigger a chronic process, subclinical rejection continues despite immunosuppression treatment or alternative pathways are involved.

The best evidence for identifying CAV as a partially inflammatory disorder comes from observations relating CRP to the presence of CAV. CRP is produced by hepatocytes as an acute phase protein, stimulated by IL 6 and is a sensitive indicator of acute and chronic inflammation in many different conditions. It is used routinely in clinical practice to monitor inflammation and infectious complications. CRP has been identified as an independent risk factor in non-transplant atherosclerosis and has been shown to be a predictor for the development of CAV after transplant, is associated with endothelial activation, increased ICAM1 levels and angiographic evidence of CAV.(70,111,126) A study of the inflammatory response in male heart transplant recipients demonstrated a significant increase in serum TNF alpha, Interleukin-10, MCP-1, and IL 8 when compared to controls. Markers of endothelial cell activation (ICAM-1, V-CAM-1) were also elevated. Patients in this study were also treated with pravastatin which reduced cytokine levels, returning to elevated levels with termination of statin treatment, highlighting the anti-inflammatory properties of statins and a potential explanation for their role in reducing the progression of CAV.(91,110)

It is clear from the literature that inflammation is considered to be an important part of not just CAV but also other forms of vasculopathy, notably non-transplant atherosclerosis. Investigation of paediatric recipients' inflammatory phenotype might provide further insight into the understanding of the aetiological processes causing CAV.

### 4.1.2 Endothelial call activation

I have alluded to the pivotal role the vascular endothelium plays in the development of CAV and its involvement in the early triggers and progression of disease (section 1.4.3). Inflammatory cells are of course the major contributors to the pathological response and require a process through which these cells can be localised to the target area, and be migrated to the appropriate tissue, in this case the donor coronary artery vascular endothelium.

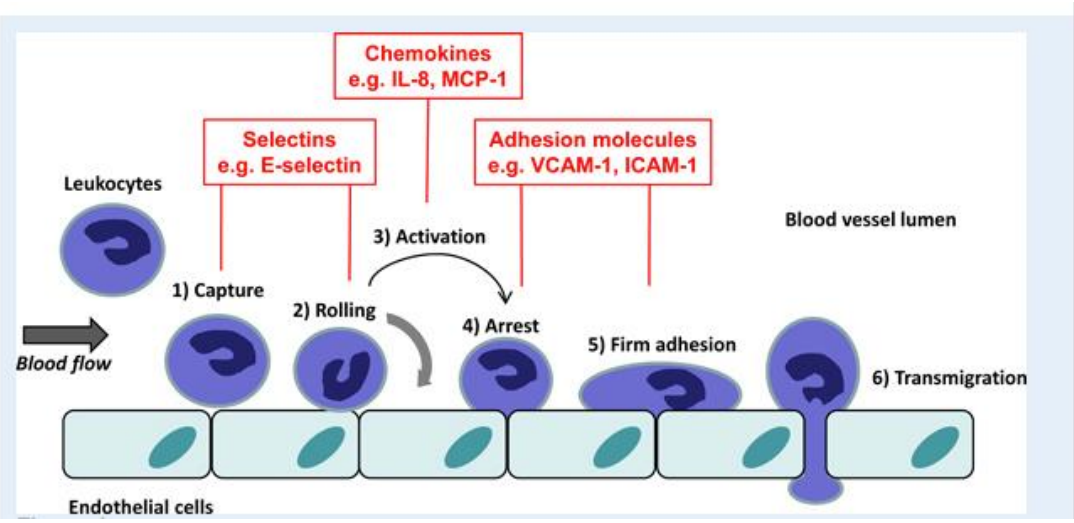


**Figure 4.1. Leukocyte adhesion and transmigration**

Proinflammatory signalling results in the increased expression of adhesion molecules such as E-selectin on endothelial cells, which facilitates capture of the leukocytes to the vessel wall. Chemokines secreted by endothelial cells activate leukocyte integrins, a process that promotes firm adhesion between leukocytes and endothelial cells via integrin-adhesion molecule interactions. Adherent leukocytes subsequently transmigrate through the endothelium to the underlying tissue. E-selectin, endothelial selectin; IAM-1, intercellular adhesion molecule-1; IL 8, interleukin-8; MCP-1, monocyte chemptactic protein-1; VCAM-1, vascular cell adhesion molecule-1.

Adhesion molecules are proteins present on the endothelium and are characterised as selectins (E selectin, P selectin) and integrins (VCAM-1, ICAM-1). Proinflammatory signals lead to increased endothelial expression and passing inflammatory cells are activated and captured from the circulation resulting in adhesion and transmigration. This process is crucial to enable a localised inflammatory response to be effective.

The immune mediated inflammatory response present in the endothelium and associated with the histological diagnosis of CAV is mediated through this biological mechanism. Increased expression of VCAM-1 and ICAM-1 have been identified in biopsy specimens in association with both acute rejection and the development of CAV. Increased serum levels of both selectins and integrins have been identified in adult transplant recipients.(22,110,113) It would appear that this component of the endothelial response to immune mediated attack is crucial to our understanding of the development of CAV and should be further investigated as part of this study.

### 4.1.3 Natural anticoagulant pathways

In response to injury the vascular endothelium attempts to maintain a largely vasodilatory, antithrombotic and anti-inflammatory phenotype in order to maintain organ perfusion. The routine use of endomyocardial biopsy as a screening method for the presence of ACR provides an essential tool for determining the molecular processes involved in the development of CAV. As we have become increasingly adept

at preventing ACR, the number of biopsies performed late after transplant has been reduced. This limits the tissue available for investigating the process involved in CAV development directly.

Following the early success of heart transplantation with the introduction of ciclosporin, clinicians realised that CAV, or vascular rejection as it was then termed, was a significant problem limiting the survival of transplant recipients. Investigators focused on the microvascular endothelium in an attempt to investigate the post transplant endothelial immune response and determine responses identifying those recipients at most risk of developing CAV. The findings of this early research have pervaded through the transplant community's investigation into CAV, though seems to have taken a secondary role to investigation into the components of the acquired immune response, and its control with the use of immunosuppressants. The importance of natural anticoagulant pathways and prevention of athero-thrombotic complications in the microvascular has featured less in terms of CAV aetiology.

This focus on the allograft microvasculature centered around an observation that deposition of excessive fibrin, representing the final product of the coagulation cascade, in the microvasculature of transplanted hearts is associated with a poor graft survival and a tendency to develop CAV. Fibrin deposition early after transplant is indicative of myocardial injury and is a poor prognostic sign.(127,128) Interestingly the presence of these findings is often observed in the absence of an inflammatory cell infiltrate suggesting an alternative pathway to cell mediated immune attack.(115) This alternative pathway is likely to be responsible for thrombus formation and fibrin deposition.

Controlling excessive thrombosis is an important function of the coagulation cascade. There are two natural anti-coagulant pathways active in the donor heart. The first is the TM-protein, protein-c and protein-s pathway present on the endothelium of arteries, veins and capillaries. During acute rejection episodes TM is down regulated in the endothelium. The second is the heparan sulfate proteoglycan-antithrombin complex which is found on arterial and arteriolar SMC, arterial intima and the venous endothelium.

Disruption of these natural anticoagulation pathways after transplant may lead to increased thrombosis and tissue damage as a result. The absence of antithrombin, a natural anticoagulant, in the cardiac allograft within the first three months has been shown to be a significant prognostic factor for the future development of CAV and is associated with subsequent graft failure.(77) Allografts that lose their ability to bind

antithrombin within the first three months after transplant are able to recover the ability to bind antithrombin. Recovery reduces the incidence of graft failure and reduces the risk of developing CAV in the future. This mechanism of loss of antithrombin is not associated with cellular infiltration typical of the acquired immune response. The trigger for antithrombin loss and fibrosis is likely to be related to IRI experienced either before, during or after implantation. The distribution of antithrombin binding activity is different following recovery with antithrombin found in venous capillaries, not present in normal hearts. VEGF is thought to be important in this neovascular response.

The additional natural anticoagulant pathway involves the endothelial TM protein. TM is an endothelium bound protein that plays a crucial role in maintaining vascular integrity through interaction with a complex multi-molecular system involved in immunity, inflammation, coagulation and cell proliferation. TM represents the molecular mechanism through which coagulation, inflammation, proliferation and complement activation can be closely linked. Thrombin binding to TM activates protein C, inactivating coagulation factors Va and VIIIa, producing an anticoagulant feedback mechanism, preventing excessive coagulation. Its biological activity is not limited to a role as a natural anticoagulant. TM acts as an anti-inflammatory molecule via both its stabilisation on the pro-inflammatory effects of thrombin and also by increasing the anti-inflammatory effects of APC. In brief, thrombin is chemotactic for monocytes and neutrophils, increasing IL 1 beta and TNF alpha neutrophil chemotaxis and this effect is blocked by TM. Thrombin effects inflammation via activation of protease activated receptors (PAR), however when bound to TM, activation is suppressed and the inflammatory response reduced.(129)

The TM lectin-like domain warrants further discussion with respect to its biological activity. Transgenic mice, lacking in the lectin like domain, have an exaggerated response to sepsis and respond with more tissue damage after IRI.(129) Lectin domain deficient mice have increased neutrophil adhesion to the endothelium and enhanced expression of adhesion molecules ICAM-1 and VCAM-1. It is likely that that the lectin domain of TM attenuates inflammation by interfering with intermediary proteins, particularly high mobility group box 1 (HMGB1) and the Lewis Y antigen of gram-negative bacteria.(130) HMGB1 is present on endothelial cells and is sequestered by TM, preventing it from binding to cell surface receptors such as Receptor for Advanced Glycation End products (RAGE) and Toll like receptors. HMGB1, through RAGE, induces cell signalling to cause angiogenesis, inflammation and cell proliferation as well as activation of the promoter NF $\kappa\beta$, leading to further cytokine release and immune activation. TM is responsible for attenuating this response.(131) This process is

important in controlling the innate immune response that is part of the early immune response immediately after transplant. Failure to control the coagulation response leads to further inflammation and fibrin deposition. This process is linked with graft failure and CAV.(132)

Further evidence for the importance of TM in the development of intimal hyperplasia comes from non-transplant atherosclerosis. Plasma TM concentrations are inversely related to coronary atherosclerosis risk and individuals with mutations in the TM gene promoter have an increased risk of myocardial infarction.(133) TM has been shown to reduce the extent of neo-intima formation in a mouse model of vascular remodelling after carotid artery ligation.(134) Recombinant TM bound to ePTFE stents significantly reduces tissue hyperplasia associated with in-stent stenosis.(135) TM therefore is implicated in neo-intima formation and vascular remodelling and as such its protective role in preventing intima-medial hyperplasia after heart transplantation is plausible.

TM is upregulated by agents known to improve endothelial dysfunction and confer a lower risk of atherosclerosis, such as statins.(136) TM also interferes with complement activation that contributes to atherosclerosis as well as increasing APC activity. Low levels of APC are known to correlate with increased atherosclerosis severity.(129)

Evidence also suggests that TM may also be protective against the toxic effects of calcineurin inhibitors (ciclosporin/tacrolimus) on endothelial cells.(44) Endothelial cells exposed to ciclosporin demonstrated signals of apoptosis (Annexin V positivity). In vitro real time PCR showed that TM gene expression and protein formation were significantly reduced in endothelial cells exposed to ciclosporin. Interestingly, the concentration of TM in the culture medium was increased, suggesting that TM on the endothelial cell surface was cleaved when these cells underwent apoptosis. When endothelial cells were cultured with both recombinant TM and ciclosporin, the percentage of apoptotic cells was reduced by 50%. The consequence of apoptosis is endothelial vascular permeability, which is significantly reduced in the presence of recombinant TM. Investigators replicated these results in the presence of tacrolimus exposure. Recombinant TM also reduced the dephosphorylation of endothelial nitric oxide (NO) synthase, increasing NO production and potentially restoring endothelial function.

The wealth of evidence highlighting TM as an important protein in vascular health warrants renewed efforts to determine is role in the development of CAV after heart transplantation.

This chapter is focused on establishing the presence and severity of CAV in our cohort

of patients. This is achieved by using IVUS to detect thickness in the intima-media layer along the length of the left coronary artery. These data can then be used to measure the severity of CAV.

Previously in chapter 3 we have established the presence of inflammation in the form of increased serum levels of IL 6, endothelial activation in the form of raised ICAM1 and VCAM1 serum levels and an increase in TM levels after transplant.

We will explore the overall severity of CAV present in our cohort using the Stanford Score and investigate any relationships between transplant demographics and clinical parameters before exploring the relationship between serum biomarker levels and CAV severity. Discussing the differences in serum levels for those patients with severe disease might provide further mechanistic information on the processes involved in the development of CAV in children. As discussed in the methods section those patients with the most severe disease were defined as having a mean IMT in the coronary artery > 0.5 mm in line with others previous work identifying this as a marker of severity likely to predict cardiovascular events.(47,104,105) Analysis is performed based on both linear relationships and by dividing patients into two dichotomized groups, the first group with a significant increase in the intima-medial layer of the coronary artery (mIMT ≥ 0.5 mm) and the second with thickening below this level (mIMT < 0.5 mm). Throughout this discussion we will consider the first to group to have CAV and the second to be CAV naïve, though of course this is a linear variable dichotomized at a clinically significant point.

## 4.2 Aims

- To outline the severity and distribution of CAV in our patient cohort using IVUS.
- To investigate risk factors for CAV severity in our patients as part of demographic and clinical data
- To determine the relationship between severity of CAV and serum levels of cytokines, endothelial activation and coagulation

## 4.3 Results

### 4.3.1 Severity of CAV

#### 4.3.1.1 Severity as defined by Stanford Grade

A mean distance of 27 mm (SD ± 8.8 mm) of main stem and left anterior descending

coronary artery was analysed per patient. Table 10. shows a comparison of characteristics between the two groups based on the degree of intimal hyperplasia. The majority of the IVUS studies demonstrated moderate or severe CAV as defined by Stanford Grade, minimal disease (Grade I) was seen in 5 (10%), mild disease (Grade II) in 3 (6%), moderate (Grade III) in 27 (56%) and Severe (Grade IV) in 13 (27%).

#### 4.3.1.2 Relationship between severity of CAV and age at transplant, time from transplant, age at study,

Scatter plots to illustrate the relationship between age at transplant, time from transplant and age at study with the degree of CAV are shown in Figure 4.2 below. The scatter plots do not demonstrate a relationship between any of these variables and severity of coronary disease. Regression analysis was used to determine any statistical relationship between clinical data and severity of CAV using mean IMT as a continuous variable. No correlation is detected for age at transplant, time from transplant and age at study.

#### 4.3.1.3 Severity of CAV and increasing donor age

Analysis of the relationship between CAV severity and increasing donor age is demonstrated as a scatter plot in Figure 4.2. Correlation coefficients demonstrate a poor correlation with wide confidence intervals. Based on linear regression analysis, increasing mIMT is related to increasing donor age (β 0.3, p 0.04) albeit with a low level of correlation. When donor age is compared between those patients in the group with CAV and those without no significant difference exists between the two groups. No correlation was identified as oart of this analysis. (Table 10)

**Table 10. Clinical parameters based on groups of CAV severity**

| | Mean IMT < 0.5 mm | | Mean IMT > 0.5mm | | |
|---|---|---|---|---|---|
| | N | Value | N | Value | p |
| Mean IMT (mm) | 39 | 0.30 (0.25 to 0.37) | 9 | 0.84 (0.64 to 94) | |
| Age at transplant (years) | 39 | 9.0 (4.9) | 9 | 9.2 (4.7) | 0.38 |
| Time post transplant (years) | 39 | 5.1 (3.5) | 9 | 5.9(3.9) | 0.91 |
| Donor age (years) | 39 | 17 (12.4) | 9 | 25.3 (17.1) | 0.12 |

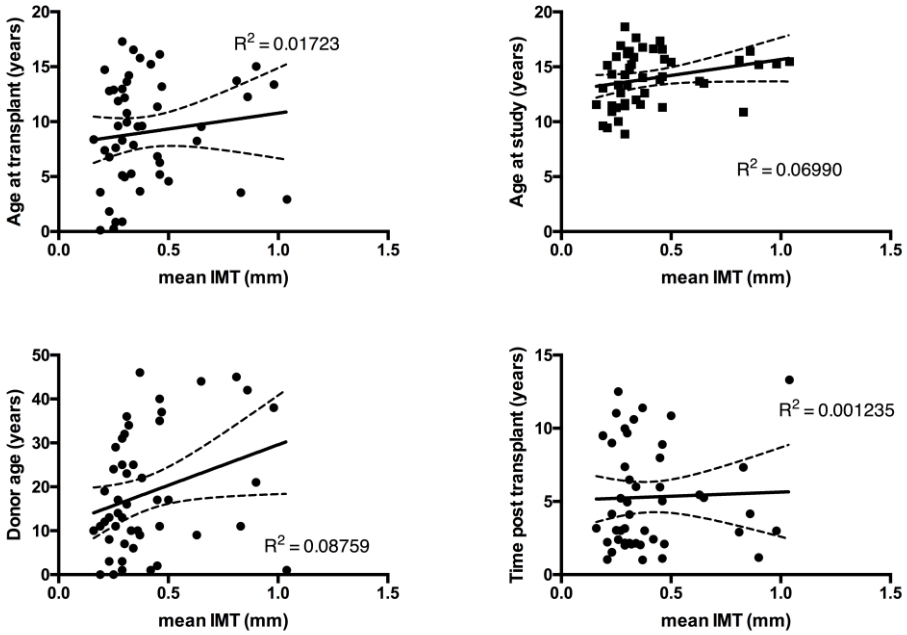


**Figure 4.2 Scatter plots of clinical parameters and mean IMT**

### 4.3.2 Relationship between cytokine/endothelial activation levels and degree of CAV

In order to understand the overall relationship between increasing severity of CAV and serum cytokine markers scatter plots were generated and can be reviewed below (Figure 4.3 and 4.4). Serum levels were compared between those patients with severe CAV and those without as defined by a mean IMT level of 0.5 mm. TM levels were lower in those with severe vasculopathy (5.5 pg/ml (4.4 to 8.6) vs 7.4 pg/ml (4.6 to 11.3) p = 0.01). vWF levels were significantly raised (0.55 pg/ml (0.18 to 1.25) vs. 0.65 pg/ml (0.47 to 1.48), p = 0.01) and TNF alpha levels were lower in those with significant disease (5.2 pg/ml (4.1 to 6.6) vs 3.9 pg/ml (3.1 to 5.5), p = 0.05. (Table 12) Scatter plots are depicted in Figure 4.5. No other significant differences in cytokines were detected between the two groups.

**A linear regression analysis was run to predict the severity of CAV (mIMT) from serum cytokine and cell adhesion molecule levels. Serum levels of vWF and TM significantly predicted the value of mIMT, F(16,22) =2.23, p =0.04, R =0.79. An increasing level of vWF and a decreasing level of TM was related to increasing intima-medial thickening. Regression coefficients and standard errors can be found in Table 12.**

Table 12. Regression coefficients and standard errors for cytokine and cell adhesion molecules vs. mean IMT

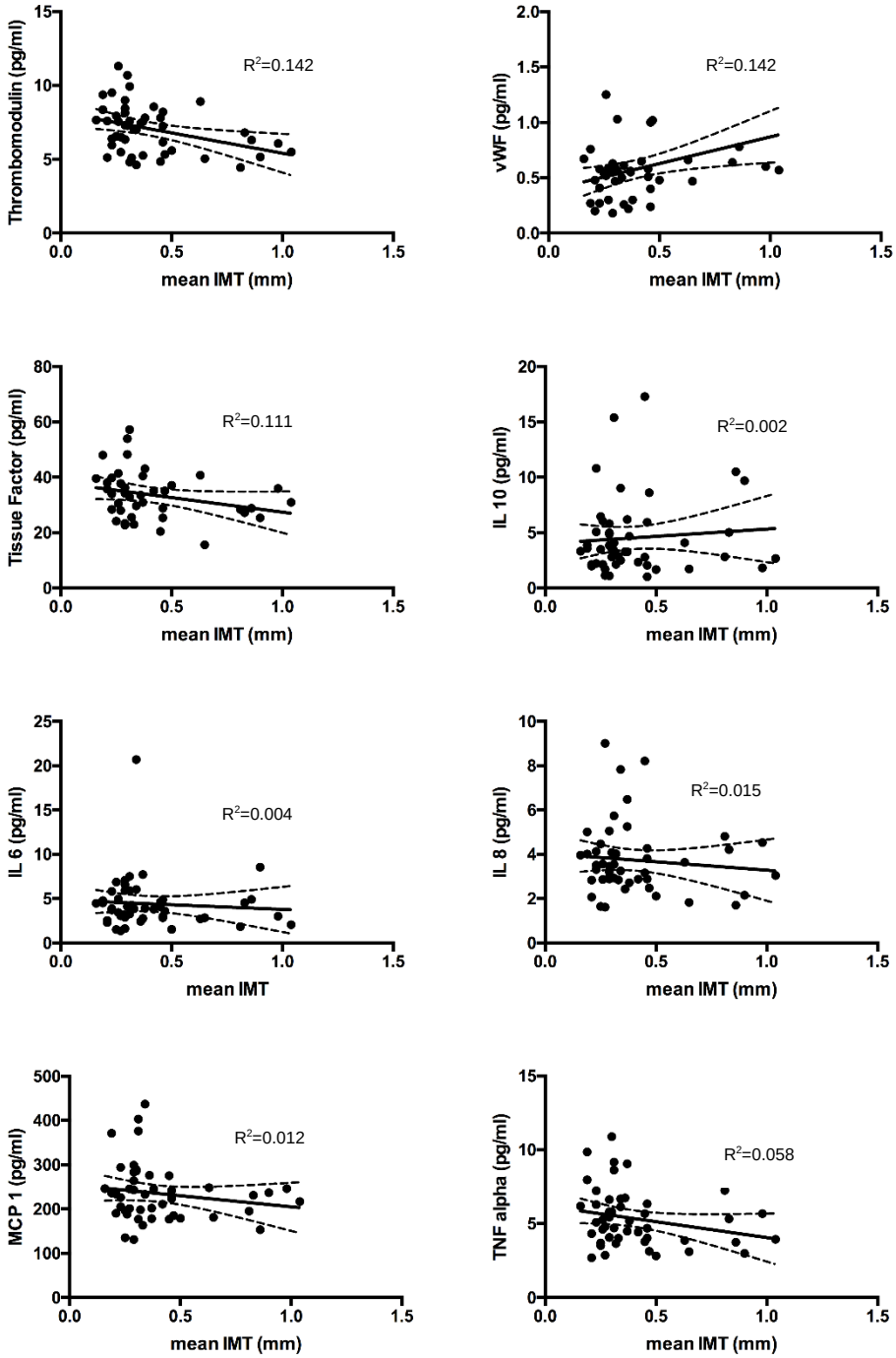


**Figure 4.3 Scatter plot of cytokine levels against intima-medial thickness (IMT)**

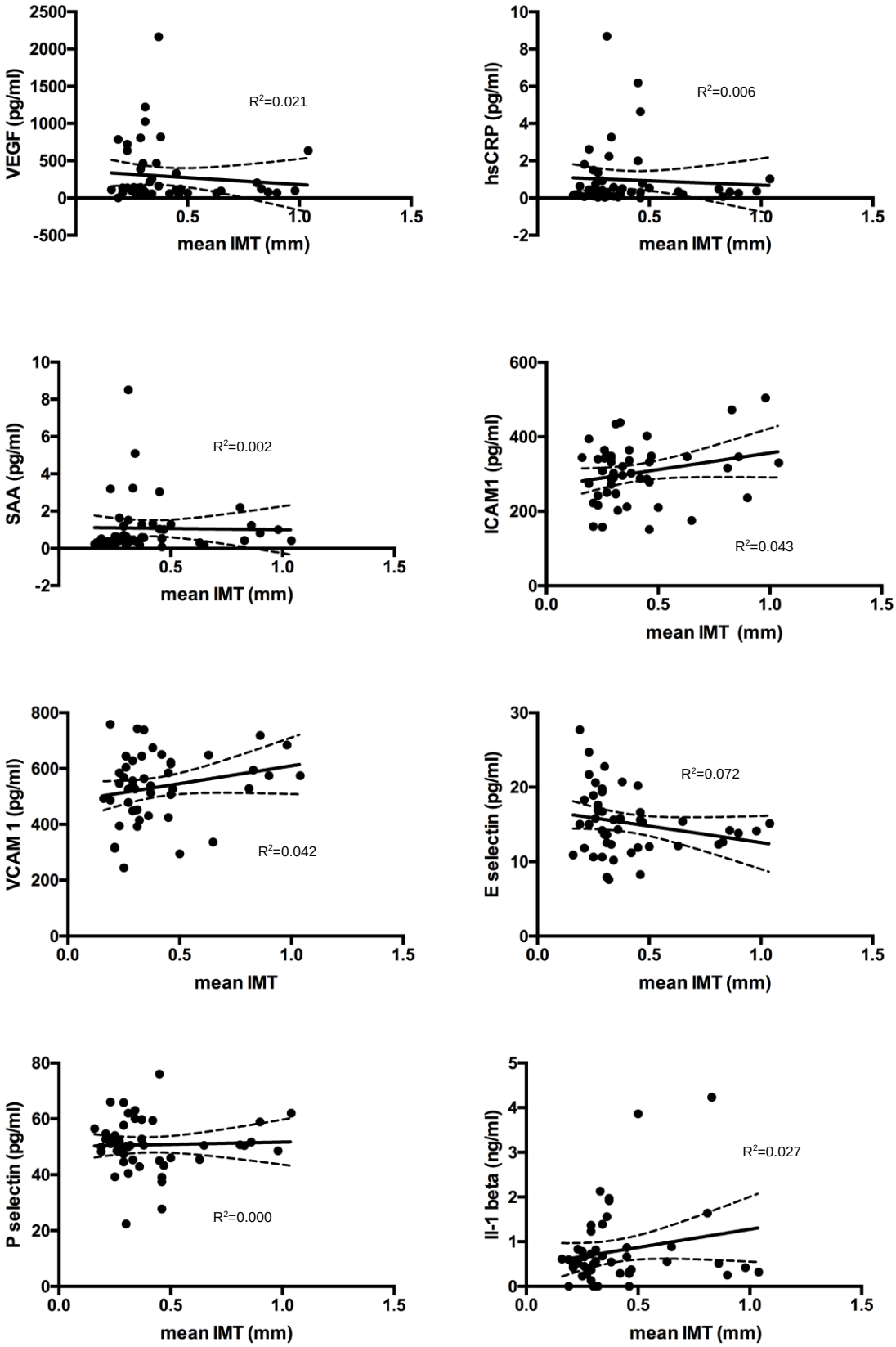


**Figure 4.4 Scatter plot of cytokine levels against intimal-medial thickness**

**Table 11. Cytokine and Adhesion molecule levels between CAV severity groups**

| | N | Mean IMT < 0.5 mm | N | Mean IMT > 0.5mm | p |
|---|---|---|---|---|---|
| mIMT (mm) | 39 | 0.30 (0.25 to 0.37) | 9 | 0.84 (0.64 to 94) | |
| vWF | 35 | 0.55 (0.3 to 0.61) | 9 | 0.64 (0.53 to 1.06) | 0.035* |
| TM | 39 | 7.5 (6.2 to 8.2) | 9 | 5.6 (5.1 to 6.6) | 0.023* |
| TNF alpha | 39 | 5.2 (4.1 to 6.6) | 9 | 3.9 (3.1 to 5.5) | 0.05* |
| IL 6 | 39 | 3.9 (3.0 to 5.8) | 9 | 2.8 (2.0 to 4.7) | 0.16 |
| E selectin | 39 | 15.6 (11.9 to 18.9) | 9 | 13.8 (12.2 to 14.7) | 0.164 |
| IL 1β | 39 | 0.54 (0.29 to 0.82) | 9 | 0.55 (0.37 to 2.75) | 0.26 |
| MCP 1 | 39 | 233 (196 to 276) | 9 | 217 (180 to 242) | 0.27 |
| Tissue Factor | 32 | 34.1 (28.1 to 39.8) | 9 | 28.9 (26.3 to 36.5) | 0.30 |
| IL 8 | 39 | 3.5 (2.9 to 4.3) | 9 | 3.1 (2.0 to 4.4) | 0.35 |
| VCAM 1 | 39 | 530 (450 to 616) | 9 | 574 (432 to 666) | 0.39 |
| VEGF | 39 | 121 (65 to 464) | 9 | 94.8 (68.4 to 163.8) | 0.42 |
| ICAM 1 | 39 | 302 (248 to 344) | 9 | 330 (223 to 409) | 0.59 |
| IL 10 | 39 | 3.49 (2.22 to 5.81) | 9 | 2.8 (1.8 to 7.4) | 0.74 |
| P selectin | 39 | 50.5 (45 to 56.5) | 9 | 50.5 (47.3 to 55.3) | 0.84 |
| hsCRP | 39 | 0.34 (0.13 to 1.36) | 9 | 0.34 (0.23 to 0.51) | 0.87 |
| SAA | 39 | 0.56 (0.37 to 1.2) | 9 | 0.82 (0.36 to 1.27) | 0.88 |

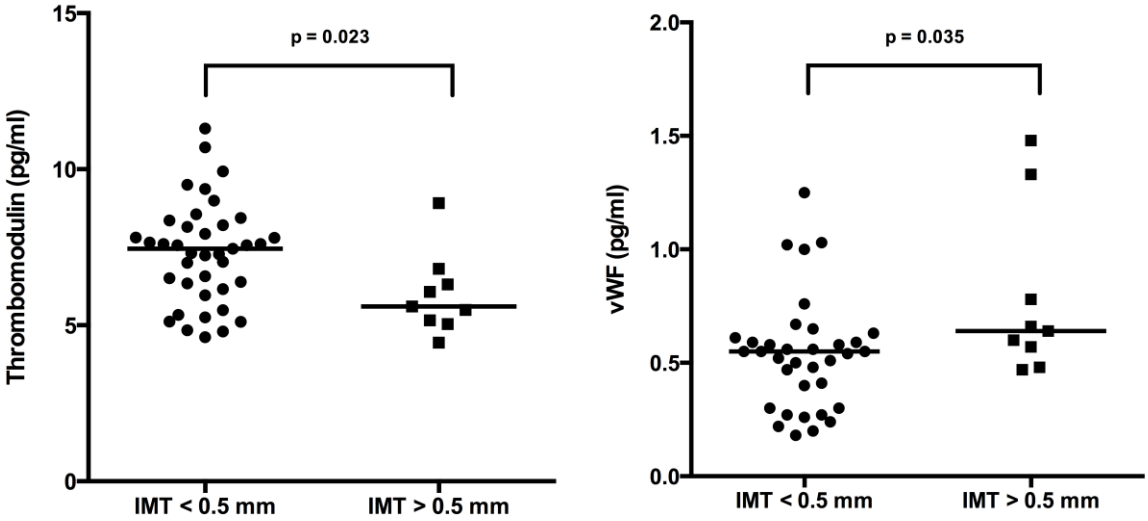


**Figure 4.5. Scatter plots demonstrating the difference in cytokine levels between severity of intima-medial thickening (IMT). vWF, Von Willebrand Factor**

**Table 12. Regression coefficients and standard errors for cytokine and cell adhesion molecules vs. mean IMT**

| Variable | B | $SE_B$ | β | P value |
|---|---|---|---|---|
| Intercept | 0.341 | 0.243 | | .175 |
| vWF | 0.293 | 0.119 | 0.388 | .022* |
| TM | -0.049 | 0.023 | -0.363 | .050* |
| MCP 1 | 0.002 | 0.001 | 0.502 | 0.053 |
| IL 6 | -0.034 | 0.017 | -0.455 | 0.059 |
| VCAM 1 | 0.001 | 0.000 | 0.441 | 0.073 |
| VEGF | 0.000 | 0.000 | 0.270 | 0.146 |
| Tissue_Factor | -0.007 | 0.005 | -0.293 | 0.182 |
| TNF alpha | -0.033 | 0.024 | -0.292 | 0.188 |
| E selectin | -0.011 | 0.009 | -0.220 | 0.205 |
| IL 1β | 0.048 | 0.041 | 0.188 | 0.258 |
| SAA | -0.041 | 0.036 | -0.273 | 0.269 |
| IL 8 | -0.020 | 0.026 | -0.148 | 0.449 |
| ICAM1 | 0.000 | 0.001 | 0.149 | 0.521 |
| IL 10 | 0.009 | 0.014 | 0.143 | 0.530 |
| hsCRP | -0.009 | 0.033 | -0.070 | 0.784 |
| P selectin | 0.000 | 0.004 | 0.020 | 0.909 |

Notes. *p<0.05, B, unstandardised coefficient, $SE_B$, standard error B, β, standardised coefficient.

## 4.4 Discussion

My results regarding serum TM levels are particularly intriguing. While TM levels were elevated in transplanted children compared to controls, significantly lower levels were demonstrated in children with the most severe CAV. This seemingly paradoxical finding mimics previous work in nontransplant atherosclerosis. High levels of serum TM were

detected in individuals with risk factors for disease, but demonstrated an inverse relationship with incident coronary artery disease.(121) This paradox might be resolved by considering that TM is a protein protective against vascular disease; in normal individuals, without any significant damage to the endothelium, levels would not be elevated. In patients at risk of atherosclerotic disease, levels would be increased due to its protective role. Low levels may reflect a disorder of this homeostatic response, preventing patients from benefiting from its ability to moderate damage, leading to progression of vasculopathy.

In order to understand our results following transplant, we need to consider TM activity at the endothelial level within the allograft. During rejection episodes the expression profile of cytokines and adhesion molecules on cardiac biopsy specimens demonstrates an increase in IL 1β, TNF-α, ICAM1, and vWF and reduction in TM and anti-thrombin III. TM is present on the endothelium in heart biopsy specimens prior to transplant and its presence is preserved after transplant in the absence of acute rejection. Infiltration of macrophages into the endothelium downregulates both TM and anti-thrombin III, leading to a disruption of natural anticoagulant pathways and deposition of fibrin. Fibrin deposition on biopsy is predictive for the later development of CAV even when identified on biopsy within the first few days after transplant .(113) Assuming that a reduction in serum levels of TM reflect a lack of TM presence on the endothelium, reduction of serum TM levels and increase in vWF in the allograft might explain why the patients with severe CAV reflected these findings in serum samples. We did not perform biopsies on our patients due to the risk and ethical considerations involved, however this is an important limitation.

Biologically, the multiple functions attributed to TM make this endothelium bound protein an important candidate for moderating the endothelial response to injury. The TM protein spans the endothelial membrane and controls biological responses to coagulation, inflammation, smooth muscle cell proliferation, and complement activation.(137) Thrombin binding to TM, produces APC, via a vitamin K-dependent anticoagulant serine protease, preventing excessive coagulation. TM acts as an anti-inflammatory molecule by stabilizing the pro-inflammatory effects of thrombin and by increasing the anti-inflammatory effects of APC. Thrombin is chemotactic for monocytes and neutrophils, increasing IL 1β and TNF-α neutrophil chemotaxis and promoting an inflammatory response; TM blocks this effect.(129)

Our patients with severe CAV also had raised levels of vWF and a tendency toward older donors. The presence of asymptomatic lesions with IMT > 0.5 mm is common, with a 59% incidence in the fourth decade.(9) vWF is synthesized by the endothelium

and platelets and is an essential mediator in promoting platelet adhesion to the endothelium.(138) Elevated levels of vWF during acute rejection have been described in adults post–heart transplantation.(22,110,139) vWF levels have also been correlated with CAV severity by IVUS, have higher levels than controls, and the potential to predict CAV development.(71,140) This provides further evidence for the importance of endothelial damage and impairment of natural anticoagulation in the development of CAV. Our association between vWF levels, older donors, and increase in IMT can be explained through these mechanisms.

It is also important to consider whether our findings could be used to predict or detect the presence of CAV. The search for serum biomarkers is important, as our “gold standard” methods of detecting CAV are reliant on coronary angiography and IVUS, which in children involves general anesthetic and rare though significant complications. The scatter plot showing the relationship between increasing coronary IMT and serum levels for significant cytokines does not suggest that levels could be used to predict the presence of severe CAV. However, the data are interesting with respect to identifying important pathophysiological processes and fit into a hypothesis that inflammation, endothelial activation, and disruption of natural anticoagulation leads to more severe CAV.(113)

TM has been relatively ignored in the transplant literature in recent years and with the availability of a recombinant TM protein, investigation into the potential as a therapeutic intervention is warranted.(141,142)

## 4.5 Limitations

A limitation of our study is the absence of biopsy specimens concurrent with blood sampling for serum markers of inflammation and cytokine activation. We did not feel that performing additional biopsies outside our protocol could be clinically justified. This prevented us from assessing the presence of cellular or antibody-mediated rejection, which might influence serum cytokine and adhesion molecule levels at the time of sampling.

## 4.6 Conclusions

These data demonstrates evidence for inflammation and endothelial activation in paediatric heart transplant recipients, suggesting that these mechanisms promote CAV. Our results also demonstrate that while circulating TM is increased after heart transplantation, patients with worse CAV have lower levels. TM has not been identified as an important factor in CAV development before. These intriguing results reinforce

the role the protective anti-inflammatory and antithrombotic mechanisms of TM might play in abrogating endothelial damage, preventing the development of CAV perhaps both early and late after heart transplantation. These data reinforce findings linking inflammation, endothelial activation, and vascular thrombosis in adult transplant patients and place natural anticoagulant pathways at the centre of discussions, hitherto unrecognised, in the aetiology of CAV.

We have found significantly higher levels of vWF and significantly lower TM levels in those patients with severe CAV. This finding is important when we consider the results presented in chapter 3 where TM levels were higher in patients after transplant than in healthy controls.

These results regarding serum TM levels are particularly intriguing. While thromobodulin levels were elevated in transplanted children, significantly lower levels were demonstrated in children with the most severe CAV. This seemingly paradoxical finding mimics previous work in non-transplant atherosclerosis. High levels of serum TM were detected in individuals with risk factors for disease, but demonstrated an inverse relationship with incident coronary artery disease.(121) This paradox might be resolved by considering that TM is protective against vascular disease; in health, levels would not be elevated, but in patients at risk of atherosclerotic disease, levels would be increased perhaps due to its protective role. Low levels may reflect a disorder of this protective response, preventing patients from benefiting from TM's role in moderating endothelial damage and leading to progression of CAV.

In order to understand these results, following heart transplantation, we need to consider the role that TM activity plays at endothelial level within the cardiac allograft. The literature suggests that during rejection episodes, the expression of cytokines and adhesion molecules on cardiac biopsy specimens shows an increase in IL 1$\beta$, TNF$\alpha$, ICAM1 and vWF and reduction in TM and antithrombin III.(22) TM is present on the endothelium in heart biopsy specimens before transplant and presence is preserved in the absence of acute rejection. Infiltration of macrophages into the endothelium down-regulates both TM and antithrombin III, leading to a disruption of natural anticoagulant pathways and deposition of fibrin, a process predictive for later development of CAV even within the first few days after transplant.(113) These findings discovered by examining biopsy samples mirror our results in the serum and might explain our findings. We know that an increase in documented rejection episodes increases the risk of CAV potentially related to the down regulation of TM in the cardiac allograft and the protective effect that TM and APC can have on the endothelium.

This hypothesis is further supported by research that suggests that during a quiescent phase, without endothelial activation, the endothelium is protected by membrane bound TM that blocks interaction with circulating leukocytes. Once the endothelium becomes inflamed the TM molecule is cleaved by proteases and the ectodomain is released into the bloodstream. The reduction in intact membrane bound TM leads to reduction in production of the protective effects of APC. ICAM-1 and VCAM-1 expression increases on the endothelium and facilitates the binding and transmigration of leukocytes leading to further inflammation and endothelial activation. Therapeutic administration of recombinant TM, encompassing the entire ectodomain, EGF domain or the serine-threonine rich region all have anti-inflammatory properties and suggest that a reduction in the serum levels of TM might be associated with increased endothelial inflammation.

### 4.6.1 Evidence to support TM as a moderator in CAV

Based on our knowledge TM appears to be an important candidate protein for moderating the endothelial response to injury. The TM protein spans the endothelial membrane and controls the biological response to coagulation, inflammation, smooth muscle cell proliferation and complement activation.(137) Thrombin binding to TM produces APC, preventing excessive coagulation. TM acts as an anti-inflammatory molecule by stabilising the pro-inflammatory effects of thrombin and by increasing the anti-inflammatory effects of APC. Thrombin is chemotactic for monocytes and neutrophils, increasing IL 1β and TNF-α neutrophil chemotaxis; this effect is blocked by TM.(129)

Patients with severe CAV also had raised levels of vWF and a tendency towards older donors. The presence of asymptomatic lesions with IMT > 0.5 mm is common with a 59% incidence in the fourth decade.(9) vWF is synthesized by the endothelium and platelets and is an essential mediator in platelet adhesion.(138) Elevated levels of vWF with acute rejection and in comparison to healthy controls in adults post heart transplantation has been described.(22,110,139) vWF levels have also been correlated with CAV severity by IVUS, have higher levels than controls and the potential to predict CAV development.(71,140) This provides further evidence for the role endothelial damage and impairment of natural anticoagulation systems play in the development of CAV. Our association between vWF levels, older donors and an increase in IMT can be explained through these mechanisms.

Further research is required into the protective role that TM might play in the development of CAV after heart transplantation. This will be expanded upon in the summary and future directions chapter.

# 5 THE EFFECT OF HEART TRANSPLANTATION ON THE NATIVE VASCULATURE

## 5.1 Introduction

The role of the vascular endothelium extends far beyond the maintenance of blood flow and is responsible for protecting the vasculature from damage. Damage to the endothelium and exposure of the underlying matrix to cells in the blood leads to inflammation and thrombosis. Derangement in lipid or sugar metabolism leading to hypercholesterolaemia or diabetes has been associated with the development of cardiovascular disease. Chronic infections, such as HIV, and autoimmune disorders commonly lead to an increase in cardiovascular events.(143) The excessive production of reactive oxygen species, superoxide ions is associated with these conditions and has a negative effect on the endothelium through a reduction in the bioavailability of NO.(144) The pathophysiologic development of atherosclerosis in these conditions is linked to endothelial damage and is akin, though not identical, to the development of CAV.

The development of vasculopathy associated with these conditions is part of the chronic course of the disease. Our ability to understand the triggers for the secondary cardiovascular effects of these diseases has stimulated the development of non-invasive techniques that are able to detect early functional change and relate this to the development of irreversible structural change.

### 5.1.1 Endothelial dysfunction: Flow mediated dilatation (FMD)

FMD is a technique that measures arterial dilatation in response to increase in blood flow. Blood flow can be increased by either physical or pharmacological means.(145) The most convenient and frequently used assessment of arterial endothelial dysfunction was developed by Celermajer and Deanfield.(146) This technique has been used to assess atherosclerotic risk factors in the young and with appropriate quality control has been used worldwide to assess the impact of risk factors on early endothelial dysfunction. The method involves inducing hyperaemic flow by occluding arterial flow with a blood pressure cuff at suprasystemic pressure below the level of the brachial artery. An ultrasound probe combined with specialist edge detection software is then used to determine the percentage change in brachial artery dimensions in response to increased blood flow after release of the blood pressure cuff. The endothelial response to hyperaemia is thought to be NO mediated.(147) Reduction in percentage arterial dilatation from baseline represents impairment in endothelial function. This technique has been used to demonstrate endothelial dysfunction in a number of non-cardiac conditions in children as young as eight years, notably viral infections and hypercholesterolaemia.(148,149) In adults endothelial dysfunction

detected using FMD is linked to the development of carotid intima-medial hyperplasia over a six-year period in patients free of cardiovascular disease.(150) It is clear that endothelial dysfunction is an important component in the progression of cardiovascular disease and assessment of the peripheral vasculature has been validated within children and adults.(151)

There has been limited research investigating endothelial dysfunction in native vessels after transplant. It is well known that heart failure prior to transplant causes impairment of endothelial dysfunction.(152,153) After heart transplantation brachial FMD demonstrates impaired endothelial function with around 50% of patients returning to normal by a year post transplant. Those patients with endothelial dysfunction at three months have increased coronary IMT at a year.(154) The presence of endothelial dysfunction after heart transplantation is also dependent on the aetiology of cardiac failure. Patients with a non-ischaemic cause demonstrate recovery of endothelial function after transplantation whereas patients with an ischaemic cause do not.(155) A previous study investigating FMD in children after transplant demonstrated a reduction in endothelial function in comparison to controls. The authors investigated NO excretion in the urine and found no difference in this between transplant recipients and controls, speculating that other factors are responsible.(156)

Further investigation into the effect of transplantation on native vessels is required and is a focus of this thesis, correlating these data with overall vascular health and investigating any links to disease of the donor allograft.

### 5.1.2 Structural changes to the native vasculature

The presence of endothelial dysfunction is an early marker, and is associated with risk factors for peripheral and cardiovascular disease. Impairment in endothelial dysfunction is often reversible through exercise, lifestyle modifications or drug therapy. Most medications that reduce the risk of cardiovascular disease such as ACEi, betablockers and statins are able to improve endothelial dysfunction.(150) Endothelial dysfunction represents a disorder of a physiological process, leading to structural pathological change over time. The techniques used to identify pathological change and their relevance to heart transplant recipients are described.

#### 5.1.2.1 Pulse wave velocity (PWV) and carotid distensibility

PWV is an investigation used to determine arterial stiffness. Arterial stiffness and elasticity are required to dampen the pulsatile nature of the cardiac impulse and provide steady flow of blood to the capillaries. Stiffening of the vessels is part of the ageing process and is associated with disease such as diabetes, obesity and hypertension.

The various imaging techniques designed to assess the progression of peripheral atherosclerosis/arteriosclerosis have focused on blood vessels accessible via peripheral ultrasound techniques. This offers a noninvasive and convenient method of assessment. PWV has been linked to cardiac risk factors and atherosclerotic abnormalities elsewhere in the vascular tree(157). Abnormalities of PWV are present particularly in renal transplant patients and confer cardiovascular risk in these patients despite measuring the stiffness in arteries distant from the coronary curculation.(158,159) A previous study investigating PWV in paediatric patients after heart transplant identified increased arterial stiffness with increased PWV with no correlation investigating the effect of this on the development of CAV.(160) I have shown previously that increased PWV is increased after transplant and that PWV and distensibility ($\beta$ index) are related to the degree of CAV as measured by IVUS in a small cohort of patients.(161)

#### 5.1.2.2 Carotid artery intima-medial thickening (cIMT)

The carotid artery offers an accessible site to measure structural changes in the arterial tree. High resolution ultrasound is able to reproducibly measure the intima-medial layer. Like PWV, cIMT is a marker of atherosclerosis elsewhere in the vascular tree and is related to unfavourable risk factors for coronary artery disease and stroke.(162,163) Measurement in children has also been linked to increased risk of cardiovascular disease.(164) After heart transplantation increased cIMT has been observed in paediatric cohorts. The degree of thickening seemed to be related to the serum LDL/HDL ratio.(165)

Bearing in mind the relationship between these remote markers of atherosclerosis in renal transplant and to non-transplant atherosclerosis, investigating the presence of these peripheral abnormalities in paediatric transplant patients at risk of CAV is an important exercise. The potential to establish a link between these relatively non invasive techniques and the presence of CAV as determined by invasive techniques might provide a valuable resource for monitoring these patients in the future. This assessment of the native vasculature is also important to investigate whether heart transpantation has a more general effect on vascular health.

Previous chapters have identified that in our cohort of patients inflammation is present after heart transplantation and that those with severe CAV have differences in the serum profile of cytokines involved in inflammation, endothelial cell function and thrombosis. We have discussed previously the effects that inflammation has on the

native vasculature in association with diseases that have an inflammatory component; diabetes, infections and autoimmune disorders.(148,166,167)

In this chapter we will present results investigating abnormalities in the native, non-transplanted vasculature and determine whether there are significant differences in vascular health in post transplants patients in comparison to controls. In addition we will examine the evidence to understand whether those transplant recipients with severe CAV have any significant changes in their native vasculature.

## 5.2 Results

### 5.2.1 Abnormalities of the native vasculature after transplant

**Table 13. Differences in systemic vascular measurements between patients and controls**

| | Patients | | Controls | | |
|---|---|---|---|---|---|
| | N | Value | N | Value | p |
| Brachial baseline diameter (mm) | 39 | 2.9 (0.4) | 31 | 3.0 (0.5) | 0.48 |
| FMD (% change) | 39 | 9.0 (4.3) | 31 | 8.0 (3.0) | 0.25 |
| Pulse wave velocity (milliseconds) | 36 | 8.3 (1.4) | 25 | 7.2 (1.2) | 0.002* |
| Systolic blood pressure (mmHg) | 40 | 113 (10.5) | 30 | 11 (10.7) | 0.55 |
| Diastolic blood pressure (mmHg) | 40 | 69 (7.2) | 30 | 64 (8.0) | 0.004* |
| Heart rate (beats per minute) | 37 | 92 (12) | 29 | 69 (12) | 0.001* |
| Carotid IMT (mm) | 41 | 0.48 (0.05) | 20 | 0.46 (0.04) | 0.10 |
| Values are mean (standard deviation), FMD represents Flow Mediated Dilatation, IMT represents Intima-medial thickness. * significance < 0.05 | | | | | |

Significant differences were detected between children post-transplant and sex- and age-matched controls for baseline heart rate, diastolic blood pressure and PWV. No difference was detected in FMD and cIMT between patients and controls. Table 13 documents the findings and demonstrates the differences between the groups and the level of significance.

### 5.2.2 Influence of CAV on the native vasculature

Table 14 divides transplant recipients into two groups depending on the severity of

coronary artery intima-medial thickness as detected by IVUS. No difference was detected in brachial endothelial function or carotid intima-medial hyperplasia between these groups, suggesting that there is no relationship between the degree of cardiac allograft vasculopathy and abnormalities in the native vasculature.

**Table 14. Participant characteristics between CAV severity groups**

| | Mean IMT < 0.5 mm | | Mean IMT > 0.5mm | | |
|---|---|---|---|---|---|
| | N | Value | N | Value | p |
| Mean IMT (mm) | 39 | 0.30 (0.25 to 0.37) | 9 | 0.84 (0.64 to 94) | 0.00 |
| Age at transplant (years) | 39 | 9.0 (4.9) | 9 | 9.2 (4.7) | 0.38 |
| Time post transplant (years) | 39 | 5.1 (3.5) | 9 | 5.9(3.9) | 0.91 |
| Donor age (years) | 39 | 17 (12.4) | 9 | 25.3 (17.1) | 0.12 |
| Systolic blood pressure (mmHg) | 32 | 112 (10) | 8 | 114(10) | 0.18 |
| Diastolic blood pressure (mmHg) | 32 | 70 (7) | 8 | 66 (7) | 0.48 |
| Heart rate (bpm) | 32 | 92 (13) | 8 | 89 (10) | 0.29 |
| Baseline brachial diameter (mm) | 31 | 2.9 (0.4) | 8 | 3.0 (0.4) | 0.83 |
| % change post FMD | 31 | 9.4 (4.5) | 8 | 7.5(3.5) | 0.96 |
| Carotid IMT (mm) | 33 | 0.48 (0.05) | 8 | 0.50 (0.05) | 0.92 |
| IMT, intima medial thickness, bpm, beats per minute, FMD, flow mediated dilatation, | | | | | |

## 5.3

## 5.4 Discussion

We considered whether the presence of inflammation/endothelial activation impacts on functional or structural changes in the native vasculature and whether there is any relationship between the presence of severe CAV and disease in the native, non-transplanted vasculature,

Our results do not show that brachial artery FMD or carotid artery intima-medial thickness was abnormal in our cohort of patients when compared to age and sex-matched controls. Abnormalities have been shown in adult patients after heart transplant previously, however treatment with statins has been shown to improve this. Its possible that no difference was detected due to almost universal treatment with pravastatin in our cohort.(110) In addition the indications for heart transplantation differ

between adults and children. A significant proportion of adult patients require heart transplantation as a result of ischaemic heart disease. Risk factors for ischaemic heart disease include diabetes, hypertension and lipid abnormalities, all of which are recognized as having an adverse effect on markers of peripheral endothelial function.

Adult studies investigating changes in FMD after transplant have demonstrated abnormal FMD in the context of heart failure with reduced systolic function which persist early after transplant and return to 5 to 10 years post transplant.(144) The authors of this study linked the endothelial dysfunction to the presence of oxidant stress.

However, we did observe that heart rate, diastolic blood pressure and pulse wave velocity were significantly increased. Differences in both heart rate and blood pressure are not surprising and can be explained due to denervation of the graft and the use of vasoactive medication in the post-transplant period. Pulse wave velocity is a measure of aortic stiffness and has been shown to be predictive for cardiovascular events in patients with atherosclerosis.(168,169) In our cohort of patients there was no association between PWV and severity of CAV in our patients, perhaps related to small numbers. Previous publications have demonstrated an association with severity of CAV as detected by IVUS using cardiac MRI to assess PWV and distensibility. We were unable to replicate our previous findings using ultrasound to measure PWV in this study.(161)

Due to the absence of a relationship between structural and functional abnormalities of the peripheral vasculature and the severity of CAV it would not be possible to use these less invasive markers as a substitute for invasive assessment of the coronary arteries. Carotid intima-medial hyperplasia was not present in our patients when compared to the control group. The association between abnormal FMD and CAV has been speculatively described in an adult cohort.(170,171) The authors demonstrated that after heart transplantation those patients with an abnormal FMD at one-month post-transplant showed an increase in coronary intima-medial thickening at a year. After one-month abnormal FMD was not associated with worse CAV.

In general endothelial dysfunction is sensitive to biological influences over a short time period and represents the functional status of the vasculature at the time of testing. As yet a causal link or association has not been established to more long-term structural changes in the vasculature such as an increase in carotid intima-medial thickening. Further work would be required to demonstrate the benefits of peripheral artery assessment to predict the progression of CAV and is unlikely to be used clinically in this regard.

# 6 SUMMARY AND FUTURE DIRECTION

In summary there are several major findings identified in this research.

1. Cardiothoracic transplantation in children leads to subclinical inflammation as identified by raised levels of IL 6 in the serum when compared with healthy controls.
2. Increased levels of proteins responsible for the adhesion of cells involved in immunity to the endothelium of the cardiac allograft are raised in the serum after heart transplantation
3. TM serum levels are raised in children after transplant when compared to healthy controls.
4. Intravascular ultrasound is a sensitive and safe method for detecting CAV in children and provides data suitable for use as the gold standard for severity and investigative research. Analysis techniques developed in this thesis have not been used previously as a method to detect the severity of CAV in children.
5. Serum levels of TM are lower in those children with more advanced CAV. Whilst the causative association between TM and CAV remains to be determined the knowledge we have regarding the role TM plays in endothelial protection and prevention of inflammation and thrombosis makes it a likely candidate affecting the development of CAV.

These intriguing results reinforce the role protective anti-inflammatory and antithrombotic mechanisms might play in abrogating endothelial damage, preventing the development of CAV both early and late after heart transplantation. These data reinforce findings linking inflammation, endothelial activation and vascular thrombosis in adult transplant patients and places natural anticoagulant pathways at the centre of discussions into the aetiology of CAV.

It is also important to consider whether our findings could be used to predict or detect the presence of CAV. The search for serum biomarkers is important, as our “gold standard” methods of detecting CAV are reliant on coronary angiography and IVUS, which in children involves general anaesthetic and potential complications. As these data are cross-sectional rather than longitudinal, our data does not provide insight into whether any of the cytokines could be used in isolation as biomarkers, however the data are interesting as it fits well into a hypothesis that inflammation, endothelial activation and disruption of natural anticoagulant pathway leads to more severe CAV.(70)

TM has been relatively ignored in the transplant literature in recent years and with the

availability of a recombinant TM protein, investigation into the potential as a therapeutic intervention, is warranted.(124,125)

There are two main limitations experienced in this study. The first is the cross sectional design, making a discussion of the temporal changes in serum markers and changes in CAV severity impossible to comment upon. This is the first time that TM serum levels have been identified as clinically significant after transplant. If levels were measured prospectively throughout the journey both before and after transplantation, with continuous monitoring of IVUS detected changes in CAV the temporal relationship could be determined and the clinically important events along the way could be included. These events would likely include the presence of rejection episodes and further analysis of immunosuppression therapy and would help to refine the role that inflammation might play in CAV progression. The difficulties in designing this type of longitudinal study is that in general CAV takes a many of years to develop and as such we are relying on the sensitivity of IVUS to detect meaningful change.

More recently a similar technique to IVUS known as OCT has been increasingly used. The spectacular pictures produced can identify the coronary wall in greater resolution and moving to this technique might improve CAV detection and provide tha accuracy of change in CAV severity that a shorter study would mandate.(52,172)

The second important limitation relates to the lack of biopsy data incorporated into the study. Cardiac biopsy, though generally safe, is an invasive and unpleasant procedure for the majority of children to experience. In our current protocol we perform cardiac biopsies early after transplant to document the adequacy of early immunosuppression. We biopsy later if rejection is suspected. The implications for this research means that biopsy samples were not taken due to the ethical implications of performing this invasive procedure, usually under anaesthetic. Biopsy data would have provided us with cardiac tissue which would have enabled us to look at the endothelium of the allograft directly. Given the opportunity to assess the presence of membrane bound TM and adhesion protocols would have strengthened our conclusions. We are currently limited by not having robust data to link the serum levels with activity at endothelial level. For future studies serial biopsy samples would be essential to reinforce this causative link, especially as any additional research will need to be longitudinal in nature.

The limitations of our study are primarily related to lack of access to an effective source of blood and tissue samples throughout the transplant journey. The ability to perform this research would be greatly benefited by creating a tissue bank for samples on which

longitudianl research could be performed. From my research in this area it seems that the innate immune response at the time of allograft implant is important in setting up the endothelium to be either protective or destructive in terms of CAV progression. Research highlights the role of Interferon gamma in this process.(19) An additional limitation might be not including this in our panel of cytokines.

The most intriguing finding in this thesis relates to the inverse relationship between serum TM levels and severity of CAV. Whilst we have not been able to establish a causative relationship it seems reasonable to postulate that having low serum levels of a protective protein might increase your risk of developing severe CAV. Hence if we were able to supplement its bioavailability in some way, perhaps the progression of CAV for those with low levels could be attenuated.

The literature suggests several mechanisms by which the bioavailability of TM might be maintained after transplant. Statins have been shown to increase the endothelial expression of TM.(173) Calcineurin inhibitors, the principal immunosupressant agent after organ transplantation is known to increase vascular endothelial cell permeability. TM prevents this increase, further supporting the role of TM in maintaining endothelial cell integrity.(174,175) Perhaps we can extrapolate that withdrawal of calcineurin inhibitors and replacement with PSI (sirolimus or everolimus) is as important as the direct action of proliferation inhibitors on the vasular endothelium. This is potentially an area of further research.

TM has been ignored as a treament strategy in cardiac transplantation. Recombinant TM is available for human use and is used in the treatment of sepsis and as an anticoagulant in clinical scenarios.(176,177) This is primarily in the treatment of disseminated intravascular coagulopathy. The graft protective effects of TM in a murine cardiac allograft model have shown preservation of vascular structure and absence of inflammatory cells around coronary arteries in those exposed to 7 days of TM infusions at the time of transplant.(178) This result is supportive of the role TM plays in preventing allograft vasculopathy and raises a research question as to whether infusion of recombinant TM early after transplant might protect the donor coronary arteries form immune/inflammatory attack and prevent CAV development in the future.

In an extension to this model the authors performed a similar experiment in which fully MHC mismatched mice were subjected to heart transplantation with varying doses of TM infused. Integrity of the graft was preserved with increasing doses of TM and graft survival was increased. The authors identified an increase in Foxp3+ regulatory CD4+ cells suggesting that TM plays a role in recruiting regulatory T cells to the coronary

arteries.(179) Several researchers are investigating the role of regulatory T cells in prevention of transplant vasculopathy across renal and heart transplantation.(180)

The implications of this encouraging murine work, based on our findings in these data, encourages us to consider clinical translational research. This would involve treatment with TM at the time of transplant and regular follow up to determine the inflammatory response through cytokine release, examination of biopsy specimens to look for protein expression on the endothelium and IVUS or OCT detect development of CAV over time.

All through history, inspiring individuals along the way have played their part in making heart transplantation a success. It is time now time to challenge our acceptance that transplanted hearts don't last, reanalyse our firmly held beliefs and dream that improvement is possible.

Now it is time for research and technology to catch up.

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# 8 APPENDICES