HMG-CoA Reductase Inhibitors Do Not Attenuate The Inflammatory Response
Associated With Glutaraldehyde-Fixed Bioprosthetic Heart Valve Conduits

by

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ABSTRACT:

Evidence suggests that there is an immunological response of the recipient to xenograft bioprosthetic heart valves. Information on the impact of HMG-CoA reductase inhibitors (statins) and their anti-inflammatory properties on bioprosthetic valve failure remains limited. We sought to examine the efficacy of statin therapy in a rodent model of bioprosthetic valve implantation.

To mimic the human scenario, fresh or glutaraldehyde-fixed aortic valve root conduits from Lewis rats or Hartley guinea pigs were microsurgically implanted intravascularly into the infra-renal aorta of Lewis rats. The syngeneic control group consisted of a fresh rat valve conduit implanted into a rat. The xenogeneic control group consisted of a glutaraldehyde-fixed guinea pig valve conduit implanted into a rat. Treatment groups consisted of xenogeneic groups treated with either daily steroids or statins.

Overall, steroid treatment attenuated the inflammatory response observed within the xenogeneic glutaraldehyde-fixed valve conduits. Treatment with statins did not decrease this inflammatory response.

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LIST OF ACRONYMS:

Ao, aorta

AV, aortic valve conduit

CHF, congestive heart failure

ECM, extracellular matrix

ELISA, enzyme-linked immunosorbent assay

EM, electron microscopy

FFPE, formalin-fixed paraffin embedded

GF, glutaraldehyde-fixed

GFP, green fluorescent protein

GP, guinea pig

H&E, hematoxylin and eosin

HMG-CoA, hydroxymethylglutarate-CoA

IL, Interleukin

IQR, interquartile range

IVC, inferior vena cava

LDL, low-density lipoprotein

RT-PCR, reverse transcription polymerase chain reaction

SVD, structural valve deterioration

TEM, transmission electron microscopy

VHD, valvular heart disease

VIC, valvular interstitial cells

1. INTRODUCTION:

The annual rate of open-heart surgery for patients with valvular heart disease (VHD) is increasing (Figure 1).¹ Despite over 60 years of research, the ideal synthetic valve replacement prosthesis does not exist. Durability and prosthetic valve related complications remain the primary concerns when deciding between the various prostheses.²

Of particular interest are bioprosthetic (tissue) valves. Despite having the poorest potential long-term durability, they are now the most common prosthesis-type implanted in the developed world.^{3,4} As such, the patient exchanges the complications related to VHD, for those relating to valve durability, also known as prosthetic valve disease.^{5,6} Improving the durability of implanted heart valves therefore is of vital importance, particularly when considering the growing health burden of VHD.

Unfortunately, due to a variety of genetic, physiological, and environmental factors relating to patients who have undergone valve replacement, the study of this topic remains limited to observational data. Understanding and improving upon durability requires a methodical approach, which is not feasible in the clinical setting. Therefore, there is a need to bring this growing clinical bedside concern to the experimental benchside to better investigate and seek knowledge of the factors relating to poor bioprosthetic valve durability. The aim of this project is to better understand the mechanisms of bioprosthetic valve failure (notably inflammation) and if these can be modified with post-transplantation medical intervention.

2. BACKGROUND:

2.1 Valvular Heart Disease (VHD)

During an average individual's lifetime, each heart valve will open and close ~3 billion times. Despite these repetitive forces, the majority of people will not experience valve failure; a testament to the regenerative capability of valvular heart tissue. Patients who do suffer some form of valvular damage are given the umbrella diagnosis of VHD.² End-stage VHD carries the consequence of hypertrophic and / or dilated negative cardiac remodeling, ultimately resulting in a 1.5 increased likelihood of developing valvular related congestive heart failure (CHF).^{2,7-9} Currently the only definitive treatment for all types of severe VHD is open-heart surgery with replacement or repair of the diseased valve(s).

2.2 Surgical Treatment for VHD

Approximately 300,000 valve replacement surgeries are performed worldwide on an annual basis. It is the second most common and fastest growing cardiac surgical procedure performed.^{1,10} Furthermore, it is now known that the presence of even moderate VHD in conjunction with coronary artery disease is associated with a worse long-term prognosis. As such, cardiac specialists are now more frequently performing concomitant valve surgery at the time of coronary artery bypass grafting procedure.¹¹

For most cases of VHD, the native valve is excised and replaced with a synthetic prosthesis. Notwithstanding over 60 years of both private and public research, the ideal valve prosthesis, as initially proposed by Harken in 1962, continues to elude us.^{12,13} A prosthetic heart valve has yet to be proven to be as effective as a normal native valve.

2.3 Bioprosthetic Structural Valve Deterioration (SVD)

Due to the risks associated with any surgical procedure that a patient is exposed to, the first operation should be the perfect and lone operation. Despite the lack of clear evidence of superiority of one valve replacement prosthesis over another, there has been a trend towards favoring the use of bioprosthetic valves, particularly for younger individuals (<65 years of age) (Figure 2).^{3,4} Unfortunately, this increasing clinical use of bioprosthetic valves does not reflect our understanding and/or knowledge of, the various factors leading to its eventual failure.¹⁴ The only option for bioprosthetic valve failure is repeat high-risk open-heart surgery, which is an undesired outcome.

2.3.1 Bioprosthetic Valves: The Progression to Failure

Most currently commercially available bioprosthetic valves have xenograft (usually porcine aortic valve or bovine pericardium) biological tissue that is mounted on a plastic alloy framework.^{12,15,16} Void of alloy metals, bioprosthetic valves do not require lifelong anticoagulation therapy, making them more desirable in certain clinical scenarios. Xenograft valves are now the most commonly implanted prosthesis in the developed

world.^{3,4} Despite improvements in manufacturing, bioprosthetic valves continue to suffer from durability issues secondary to calcific SVD.^{2,17-20} Notably, SVD deterioration correlates highly with age at the time of implantation.²¹ The only option for bioprosthetic SVD leading to failure is reoperation at an estimated cumulative rate of ~2%/yr.¹⁷ Therefore improving the durability of bioprosthetic heart valves is an extremely important endeavor considering the growing burden of VHD.

2.4 The Role of Inflammation in Bioprosthetic Structural Valve Deterioration

2.4.1 The Link with Atherosclerosis

Current understanding of bioprosthetic SVD is in large part due to increasing knowledge of native aortic valve failure.^{2,14,22,23} Though inherently different (i.e. native tissue versus xenograft tissue), it is now known that calcification of both native aortic and bioprosthetic valve tissue leads to valve failure. Importantly, it is believed that an element of valvular calcification is associated with atherosclerosis (i.e. inflammation, lipid deposition, osteogenesis).^{2,14,24,25} Increasing evidence suggests that risk factors traditionally associated with coronary and vascular atherosclerosis, are also involved in bioprosthetic SVD.^{22,24-28} Individuals with diabetes, hyperlipidemia, smoking or a metabolic syndrome diagnosis, have been shown to have higher rates of bioprosthetic valve calcification. Additionally, explanted bioprosthetic valves that have experienced SVD demonstrate the presence of foam cells and bone regulatory proteins further implicating an atherosclerotic-like process.^{29,30} Importantly, there has been a steady

increase of these risk factors in patients undergoing cardiac surgery in Manitoba over the past decade (Figure 3).

2.4.1 Atherosclerosis: More than Just Plaque

Though atherosclerosis has been traditionally associated with frank vessel wall plaque deposition, this paradigm is not applicable to patients with VHD and bioprosthetic SVD. Indeed, plaque deposition remains the final result of a long-standing complex biochemical injury response. Endothelial dysfunction, inflammation, oxidative stress, pro-osteogenic mediator effects, in addition to patient risk factor profiles have all been implicated in this injury response long before there is visible plaque formation.³¹⁻³⁹ As such, though bioprosthetic SVD may share similar pathways and patient risk factors with plaque forming atherosclerosis, it is unknown if in valvular tissue, “traditional plaque” formation is the final result of this atherosclerosis process. Therefore viewing the atherosclerotic process in a binary fashion, i.e. with or without visible plaque, is an oversimplification of a multifaceted clinical entity. A more prudent methodology involves the analysis of the inciting steps associated with an atherosclerotic process, in particular inflammation.

2.4.2 Inflammation and Bioprosthetic Structural Valve Deterioration

The pathogenesis of bioprosthetic SVD is a complex multifactorial process involving mechanical shear stress, immune-related factors, inflammation, and subsequent calcium

deposition.^{14,40} The immune response following bioprosthetic valve implantation has been categorized into three possible processes.^{40,41} Firstly, that associated with post-surgical wound healing. Secondly, the non-specific inflammatory response to a foreign material. Finally, the immune mediated response to the xenogeneic implantation. Despite a poor understanding of these immune mediated processes, the final common pathway remains fibrocalcification.⁴¹ A better appreciation of the mediators involved in the immune response may give insight into potential therapeutic interventions.

3. HYPOTHESIS:

Bioprosthetic valves, as a result of the mandatory fixation process have altered tissue properties (i.e. loss of endothelium and nonviable extracellular matrix (ECM)) compared to native valvular tissue. Increasing evidence suggests that bioprosthetic valves elicit a recipient inflammatory response, which may influence progression to structural valve deterioration. It is hypothesized that daily treatment with HMG-CoA reductase inhibitors (also known as statins) will attenuate this recipient inflammatory response.

3.1 Hypothesis Justification: Bioprosthetic Valve Engineering and its Impact on Structural Valve Deterioration

Prior to final assembly in the manufacturing process, the tissue valve undergoes a mandatory (and usually proprietary) fixation process. Since the 1970's, manufacturers of bioprosthetic valve have used glutaraldehyde, which irreversibly cross-links tissue

proteins and valve elements in the prosthesis. Collagen cross-linking serves to “improve the hemodynamic profile of the valve”, in addition to providing added mechanical strength to the tissue.⁴¹⁻⁴⁴ Xenogeneic antigen cross-linking (i.e. masking) theoretically modifies major antigenic sites, thereby interfering with recipient antibodies.⁴⁵ As a result both the immunogenicity and subsequent thrombogenicity of the prosthesis are reduced.^{46,47} This fixation process, conversely, also creates key fundamental differences between bioprostheses and native valve tissue.

The cytotoxic glutaraldehyde fixation process has been strongly implicated in calcium mineralization as the aldehyde modification of the ECM of the prosthesis tissue results in the loss natural inhibitors of mineralization.^{48,49} As a result, with the cells made nonviable via fixation, there is membrane disruption with subsequent calcium extrusion into the ECM. With increasing concentration, calcium crystallization is initiated. Secondly, overtime without the regenerative capabilities of viable tissue, there is infiltration and degradation of important ECM proteins, collagen and elastin, with further progression of calcium mineralization and subsequent SVD.^{12,50,51} Thirdly, the pathological calcification of bioprosthetic valves leading to SVD also appears to involve proteins traditionally associated with physiological bone calcification.⁵²⁻⁵⁴ Osteopontin, osteonectin, and osteocalcin expression and activity is left unchecked without the natural inhibitors associated with an intact ECM. Finally, glutaraldehyde fixation eliminates the response to the natural dynamic motion of the tissue, thereby increasing mechanical stresses that potentiate damage to the non-regenerative ECM.^{55,56} It has been well

described that this mechanical stress on the “fixed” static tissue stimulates calcification through multiple mechanisms eventually leading to bioprosthetic SVD.^{12,57,58}

3.2 Hypothesis Justification: Bioprosthetic SVD and the Link with Atherosclerotic Inflammation

Atherosclerosis is a complex biochemical signaling process. Active lesions demonstrate significant morphological and infiltrative changes within diseased vessels.⁵⁹⁻⁶² Endothelial dysfunction, foam-cell infiltration, and ECM alterations are only a few of the changes associated with atherosclerosis.^{61,63-65} Similar changes have been described in native and bioprosthetic valve calcification.^{22,66} On the whole, atherosclerosis is now viewed as an active inflammatory process.⁶¹ It is known that inflammatory mediators, such as T-cells and macrophages, by way of promoting lipid accumulation, ECM changes, and chemotaxis, play an important role in the atherosclerotic process.^{59,67} Studies have also implicated T-cells and macrophages as important cellular mediators involved in the immune response to bioprosthetic SVD.^{40,68-73} Furthermore, it is now known that pro-osteogenic proteins, influenced by the inflammatory response, also play an important role in atherosclerosis.^{31,74,75} These proteins have also been implicated in calcification and SVD of bioprosthetic valves.^{31,52,53,74}

3.2.1 Hypothesis Justification: Patient’s Age and its Impact on Bioprosthetic SVD

Despite numerous manufacturing “anti-calcification” protocols to attenuate the aforementioned changes, one non-modifiable risk factor, patient’s age at the time of implantation, remains the strongest predictor of early SVD.²¹ Though an active immune response has been proposed, the mechanism of accelerated calcification in younger patients remains largely unknown.^{14,40} As seen in adolescents, despite the absence of traditional risk factors, the early signs of atherosclerosis (i.e. fatty streaks) are present.⁷⁶⁻⁷⁸ It is unknown if a premature inflammatory process is also involved in SVD of bioprosthetic valves implanted in patients of younger age. By examining early signs of an inflammatory process in our young rodent *in vivo* model, we will address this question.

3.3 Hypothesis Justification: Statins and Their Impact on Bioprosthetic SVD

Statins (hydroxymethylglutarate-CoA [HMG-CoA] reductase inhibitors) are a class of medications traditionally used to lower low-density lipoprotein (LDL) cholesterol levels. They have been associated with a reduction in a variety of vascular ischemic events.^{79,80} Furthermore, increasing information suggests that statins also inhibit atherosclerotic inflammatory processes independent of their lipid lowering effect.⁸¹⁻⁸⁴ As a result of these pleiotropic anti-inflammatory properties, statins are seen as having general plaque stabilization properties.^{85,86} Therefore statins are able to attenuate the atherosclerotic process via two mechanisms. Firstly, by the lowering of bad cholesterol levels, that are a key component to plaque formation. Secondly, via the inhibition of cytokines/chemokines through several immune intracellular kinase pathways (i.e. STAT-3, ERK

$\frac{1}{2}$).⁸⁷ Additionally, statins have also been shown to reduce neointimal SMC proliferation.⁸⁸ Furthermore, aggressive statin therapy treatment has shown to decrease circulating levels of pro-osteogenic proteins in patients with vascular atherosclerosis.⁸⁹ *In vitro* studies have demonstrated that statin therapy is associated with a reduction in the activity of alkaline phosphates, a marker of osteogenesis.⁹⁰ As a result of these positive anti-atherosclerotic anti-calcific properties, statins have been investigated for their ability to potentially attenuate native valve calcification.⁹¹⁻⁹⁴ Though some studies suggest that statins may be able to attenuate bioprosthetic SVD, data remains observational and limited at best.^{24,25,95}

3.3.1 Hypothesis Justification: The use of Rosuvastatin as the Agent of Choice

Statins have been in clinical use since the mid 1980s.⁹⁶ Since the introduction of lovastatin, there has been three generations of statins that have been commercially available. The most commonly known statin, atorvastatin (Lipitor ®), is a second-generation agent. Rosuvastatin (Crestor ®) is a third generation agent, and the newest statin on the market. Compared to others, it is the most effective in reducing cholesterol levels for mg/dose equivalent.^{87,97} Furthermore, as the drug is metabolized mainly through the CYP2C9 pathway (as oppose to the CYP3A4 pathway which can be inhibited by a variety of agents), makes it a promising candidate to reduce side effect profile, interaction with other drugs, and improve anti-inflammatory properties.⁸⁶ Rosuvastatin has been successfully used as an anti-atherosclerotic agent in several animal models.⁹⁸⁻¹⁰²

Finally, with regards to atherosclerotic lesions specifically, rosuvastatin appears to be superior to first generation agents with respect to lesion regression.⁹⁸

4. STUDY OBJECTIVES:

To investigate the role of inflammation leading to bioprosthetic valve failure our objectives were as follows:

1. To build upon and master a biologically valid novel *in vivo* intravascular rodent model of bioprosthetic valve implantation.
2. To ascertain the recipient inflammatory response to valve implantation using histological (hematoxylin & eosin and immunohistochemistry) staining and cytokine (serum and tissue) analyses.
3. To determine the efficacy of statin therapy to attenuate the recipient inflammatory response.

5. MATERIALS AND METHODS:

5.1 Clinical Impact: Database Query

To examine the impact of valvular heart disease in Manitoba, the Manitoba Adult Cardiac Surgery Database (Cardiac Sciences Program, Winnipeg Regional Health Authority) was queried. The proportion of patients requiring surgery for valvular heart disease in isolation, or in combination with another cardiac procedures was identified.

5.2 Study Design:

5.2.1 In Vivo Rodent Model Rationale

To mimic the clinical scenario, in addition to addressing the deficiencies of the previously published *in vivo* and *in vitro* models, a microsurgical rodent *in vivo* model was utilized from which to investigate our specific aims.¹⁰³⁻¹⁰⁶ As bioprosthetic valve implantation is a form of xenotransplantation, the experimental model incorporated the use of two different rodent species to replicate this clinical scenario. Unlike previous models that have employed a subcutaneous method to mimic orthotopic implantation, a surgically transplanted donor valve was anastomosed into the recipient infra-renal abdominal aorta, thereby creating an intravascular heterotopic transplantation model (Figure 4).

The “trade-off” with this model, though more physiologic and potentially clinically relevant, remains the quantification of changes, particularly with regards to histological tissue analysis. Furthermore, unlike *in vitro* models, a precise mechanistic pathway remains difficult to achieve with *in vivo* protocols. Therefore to methodically assess bioprosthetic SVD, each step from fixation to implantation required separate evaluation.

5.2.2 Study Cohorts:

5.2.2.1 Syngeneic Cohorts

To assess the impact of surgical wound healing, in isolation, on the morphological changes of the implanted valve, a syngeneic fresh (**Fresh**) donor Lewis rat (**Rat**) aortic valve was implanted into a recipient Lewis rat (**Rat**). This served as the surgical control arm. This cohort is represented by the color green. To assess the impact of glutaraldehyde fixation, a syngeneic glutaraldehyde-fixed (**GF**) donor Lewis rat (**Rat**) aortic valve was implanted into a recipient Lewis rat (**Rat**). The color yellow represents this cohort.

5.2.2.2 Xenogeneic Cohorts

To mimic the clinical scenario of xenotransplantation, a glutaraldehyde-fixed (**GF**) donor Hartley guinea pig (**GP**) aortic valve was implanted into a recipient Lewis rat (**Rat**). This functioned as the control experimental arm. This cohort is represented by the color red. Treatment cohorts consisted of: [1] **GF GP-to-Rat** animals treated with steroids

(represented by blue), and [2] **GF GP-to-Rat** animals treated with statins (represented by purple).

A fresh Hartley guinea pig implanted into a Lewis rat cannot be performed as this implanted valve, without glutaraldehyde fixation, would undergo hyperacute rejection with subsequent thrombosis.

5.2.3 Intervention Treatment:

To determine if the degree of morphologic changes that occurred with xenograft implantation (xenogeneic cohort) could be attenuated with medical treatment (statin therapy), the following intervention arms were tested:

5.2.3.1 Steroid Treatment

Methylprednisolone [Pfizer, Quebec, Canada] (0.5mg/kg) was administered daily via an intraperitoneal route (optimize absorption) for the entire study period. This dose represents what is commonly used within the clinical setting.¹⁰⁷ This medication served as the control “anti-inflammatory” agent to compare the efficacy of statin therapy.

5.2.3.2 Statin Treatment

Rosuvastatin [Crestor; AstraZeneca Canada Inc, Mississauga, Ontario] at a dose of

20mg/kg is representative of what has been previously reported within the literature, was delivered daily via gavage for the duration of the study period.¹⁰⁸ This medication was kindly donated by the Manitoba Cardiac Sciences Program, Section of Cardiology.

5.2.4 Surgical Procedures:

5.2.4.1 Donor Valve Harvest Operation

Inbred Lewis rats (100 to 125 grams) and Hartley guinea pigs (80 to 100 grams) (both from Charles River Laboratories [Quebec, Canada]) were used as the donor aortic valve conduit subjects. Inbred Lewis rodents are required, as compared to other less expensive rat alternatives (i.e. genetically dissimilar Sprague-Dawley rats), as a true syngeneic control arm is necessary to compare to the xenotransplanted guinea pig arm. It is known that even within seven days, a rodent allograft model will still elicit a negative host response.⁷⁰

The donors were anesthetized with inhaled isoflurane (3-5% induction, followed by 1-2% maintenance) [Lewis rats], or euthanized with pentobarbital (50mg/kg) [Hartley guinea pigs], to permit valve explantation. The aortic valve was explanted in a modified fashion as initially described by Yankah *et.al.*¹⁰⁹ Following a median sternotomy, the pericardium was opened exposing the ascending aorta. The aortic valve conduit, which was subsequently implanted with an end-to-end anastomosis technique (i.e. an interposition graft) was prepared in the following manner: [1] coronary artery ligation

using 10-0 nylon sutures, [2] trimming of the right ventricle and apical portion of the left ventricle subvalvular muscle, [3] meticulous inspection and “water” testing of the integrity of the aortic valve leaflet to ensure no injury had occurred (Figure 5).

5.2.4.2 Fixation Process

Following aortic valve harvest from the donor, the valve conduit was, [1] immediately transplanted into the recipient, or [2] fixed in 0.625% glutaraldehyde for 48 hours and then thoroughly rinsed in phosphate buffered saline (PBS) [5 minutes x 3] before transplantation, depending on the experimental arm. The glutaraldehyde concentration and fixation time were similar to what has been previously reported.¹¹⁰⁻¹¹⁴

5.2.4.3 Recipient Operation

Inbred Lewis rats (150 to 175 grams) were used as the primary recipient subjects. Younger (i.e. smaller) rodents were employed to better mimic younger individuals undergoing valve replacement. Following laparotomy, the infra-renal aorta in the recipient animal was mobilized. Following the application of distal and proximal vascular clamps, the aorta was transected and trimmed to allow for the implantation of the donor valve-conduit as an end-to-end interposition graft. Blood flow and hemostasis from the proximal aorta, through the valve conduit, to the distal aorta was confirmed upon completion of the anastomosis (Figure 5). Animals were administered post-operative analgesia, and given food and water ad lib as per our institution’s animal protocols.

5.2.4.4 Explant Operation

At the end-point of the study protocol (28 days), the recipient animals were euthanized (pentobarbital) and the implanted donor aortic valve conduit and the recipient ascending aorta were explanted for tissue analysis.

5.2.5 Study Period

Following donor valve implantation, the study period was twenty-eight days. Based on previous studies, a 28-day study period was sufficient to demonstrate negative infiltrative changes within the implanted valve.^{40,68-70} During the first post-operative week, the recipient animals underwent a baseline rodent ultrasonographic analysis. During the last week of the study period, the recipient once again underwent a follow-up ultrasonographic analysis.

5.2.5.1 In Vivo Rodent Ultrasonography

To assess the cardiac function and integrity/characteristics of the implanted valve, rodent transthoracic and transabdominal ultrasonography was performed using a 13-MHz linear probe (GE Vivid 7, Milwaukee, WI, USA). Baseline (within the first post-operative week) and follow-up (within the last week of the follow-up period) studies were performed. Cardiac function was assessed as previously described.¹¹⁵ Briefly, following

inhaled anesthesia with isoflurane, the recipient rat hearts were imaged in the 2D parasternal short axis and long axis views to obtain LV geometry indices. The implanted aortic valve conduit was imaged in the abdominal midline long and short views for valve morphology and function. The peak velocity across the AV was calculated using continuous wave Doppler. Changes between baseline and follow-up studies were documented and compared between the various cohorts. At the end of the project, a 50-MHz high-resolution probe (Vevo 2100, Visual Sonics, Toronto, Canada) was used.

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5.3 Tissue Sectioning:

5.3.1 Hematoxylin and Eosin (H&E) Staining

Following explantation, the aortic valve conduit tissue was rinsed with PBS and then immersed in 10% buffered formalin for fixation and storage. The tissue was formalin-fixed and paraffin embedded (FFPE) as outlined in Table 1. The embedded tissue was sectioned into 5- μ m sections, and H&E stained using the protocol outlined in Table 2. Two clinical pathologists scored each specimen in a blinded fashion and came to a consensus on the findings. The degree of cellularity/inflammation of the valve, media, and adventitia were scored on a four-point scale, similar to what has been previously reported (Figure 6).^{40,68-70} The scoring scheme was as follows: 0, no inflammation/infiltration (<5%); +, mild inflammation/infiltration (5-24%); ++, moderate inflammation/infiltration (25-49%); +++, severe inflammation/infiltration (50-74%); and

++++, very severe inflammation/infiltration (75-100%). Furthermore, the integrity of the media and commissural areas of the explanted valve conduit were also commented on.

5.3.2 Immunohistochemistry

Due to the necessary glutaraldehyde-fixation required for the experimental arms, FFPE sections had significant non-specific background staining. As a result, a second series of experiments were performed with the explanted tissue embedded in optimal cutting temperature compound (OCT) for subsequent frozen sections. Alongside the specimen, splenic and thymic tissues were also embedded to permit internal control when staining. Frozen sections (10-µm) were obtained of the embedded block. Tissue and slides were stored at -80 degree Celsius until required. Slides were stained following the protocol described in Table 3.

5.3.2.1 Antibodies

See Table 4.

5.3.3 Photography

Light microscopy pictures were taken with the Zeiss Axioscope 4 microscope (Carl Zeiss Canada Ltd., Toronto, Ontario) at 40-100x. Imaging analysis and quantification of positive staining was performed with Adobe Photoshop CS5 Extended. The expected

“brownish” discoloration of control rat spleen and thymus were used as a reference to define the color range representing positive staining. This color range was used to quantify positive staining on the respective experimental aortic valve conduit sections. The various percentages per specimen were averaged to determine the final percentage of the section that was antibody positive.

5.3.4 Electron Microscopy (EM)

To compare with previous findings from the literature, control (not implanted) aortic valve conduits were examined with electron microscopy. Donor aortic valve conduits destined for electron microscopy analysis were required to be processed in an alternative fashion to those for routine histology. Briefly, following sternotomy and exposure of the heart, the left ventricular apex was cannulated with a needle and osmium tetroxide / Sorensen’s phosphate buffer, was flushed through the heart to eliminate the blood and fix the aortic valve immediately. Following rapid explantation, to avoid degradation of the tissue, the valve conduit was placed in 3% glutaraldehyde / Sorensen’s phosphate buffer. Following post-fixation in 1% osmium tetroxide / Sorensen’s phosphate buffer and ethanol dehydration, the tissue was resin embedded. The embedded tissue was sectioned (50-60 nm) and underwent lead/uranium staining. After which, electron microscopy was used to take the micrographs.

5.4 Serum Cytokine Analysis:

5.4.1 Blood Collection:

5.4.1.1 Pre-Operative

Pre-operative recipient animal blood was collected at time of anesthesia prior to surgery. The R.O. Burrell animal facility (St. Boniface Research Center) performed this procedure. Following general anesthesia, the area of the jugular vein (lateral to the sternoclavicular junction) was identified and shaved. After identification with a fine (25 gauge) needle, approximately 200uL of blood was withdrawn.

5.4.1.2 Study End-Point

Following euthanasia at the study end-point, the abdominal inferior vena cava (IVC), at the level of the renal veins was identified and exposed. The IVC was entered into with a large (16 gauge) needle, and the animal was exsanguinated. Approximately 7mL of blood was collected. This was performed prior to donor valve explantation

5.4.1.3 Serum Preparation

Following collection, blood was allowed to clot for 30 minutes at room temperature. The clotted blood was centrifuged at 1,500g at 4 degrees Celsius for 25 minutes. The serum was aliquoted and stored at -80 degrees Celsius.

5.4.2 Cytokine Analysis

Cytokine analyses were performed using the Bio-Rad Bio-Plex Rat Cytokine Assay Kit[®] (Bio-Plex Rat Cytokine 9-Plex A Panel, Catalogue Number: 171-K11070). This is a multi-plex bead-based assay system designed to quantify multiple analytes (i.e. cytokines) in diverse matrices (i.e. serum). The protocol, as described by the manufacturer (Bio-Rad), was followed. In brief, the conjugated micro-beads were dispensed into a 96-well filter plate. Following three washes, 50µLs of diluted standard or sample (serum) were added per well; standards / samples were run in duplicates. The filter plate was placed on a microplate shaker, and allowed to incubate at room temperature for 30 minutes. Following three washes with vacuum filtration, 25µLs of detection antibody was added to the wells. Once again, following 30 minutes of incubation at room temperature on the microplate shaker, the filter plate was washed three times. 50µLs of streptavidin-PE was added to the wells, and the filter plate was incubated for 10 minutes on the microplate shaker at room temperature. Following three washes the beads were resuspended in assay buffer. The plate was read with the Bio-Plex 200 multiplex array system[®]. Data acquisition and cytokine concentration correlation with mean fluorescence intensity was performed using the Bio-Plex Manager 5.0[®] software.

5.5 Aortic Tissue Cytokine Analysis:

5.5.1 Tissue Collection

At the study end-point, a tissue sample from the donor valve conduit (aortic wall at the level of the sino-tubular junction, approximately 1mm x 3mm) was collected prior to the specimen being embedded in OCT. As an internal control, a similar sized sample of recipient aortic tissue was also collect. (Figure 7) Tissue was frozen and stored at -80 degrees Celsius.

5.5.2 Tissue Preparation

One-day prior to cytokine analysis, the aortic valve conduit tissue was thawed, then mechanically homogenized with a glass tissue homogenizer in 150 μ Ls of tissue extraction reagent. The tissue extraction reagent was a cocktail of: [1] InvitrogenTissue Extraction Reagent II [®] (Catalogue Number: FNN0081A), and [2] 7x concentration of the Roche protease inhibitor cocktail tablet [®] (Catalogue Number: 11-836-153-001). The 7x protease inhibitor cocktail was added to the tissue extraction reagent to create a 1x final concentration. Following homogenization, the sample was stored at -80 degrees Celsius. Just prior to cytokine analysis, the homogenized sample was thawed and centrifuged at 10,000g for 10 minutes. The supernatant was collected for both cytokine analysis and protein quantification.

5.5.3 Cytokine Analysis

Tissue cytokine analysis was performed in a similar fashion as described previously

under serum cytokine analysis. However the sample was now the undiluted supernatant following homogenization. The remaining steps were the same.

5.5.4 Protein Analysis

To standardize the quantification of tissue cytokines, protein analysis was performed on the homogenized supernatant. The Bio-Rad DC Protein Assay ® kit (Catalogue Number: 500-0116) was used for protein quantification. The protocol, as described by the manufacturer, was followed. In brief, following preparation of the standards in the tissue homogenization cocktail, 5µLs of standard or sample (homogenized tissue supernatant) were added per well in a microtiter plate. The standards and samples were run in duplicate, after which 25µLs of reagent A' followed by 200µLs of reagent B were added to each well. The plate was allowed to incubate at room temperature for 15 minutes. The absorbance, to calculate final concentration, was read at 750nm.

5.5.5 Standardization of Results

As each recipient will have a varying basal response following implantation of the donor valve, the cytokine quantification was corrected for by using an internal control (recipient aortic tissue). Firstly, cytokine concentration (pg/mL) was standardized to the protein concentration (mg/mL) for that respective tissue sample. To eliminate the basal cytokine concentration, the standardized recipient value ($\text{pg [cytokine]} / \text{mg [protein]}$) was subtracted from the donor value for each animal subject. If the obtained value was

negative, the result was recorded as zero. This calculation was performed for each animal per group, for all the measured cytokines.

5.6 Power Calculation

Unlike *in vitro* models, or *in vivo* models where the outcomes are measured quantitatively, performing a power calculation for animal numbers on semi-quantitative histological data remains difficult. Furthermore, determining the various factors required for the calculation (i.e. sample means and expected changes) is near to impossible when examining a dynamic and multifaceted process such as inflammation. Based on previous similar studies, we have determined that a sample size of 8 - 12 animals per arm should be adequate to elucidate any changes between the cohorts.^{40,68-70}

5.7 Statistical Analyses

Post-operative surgical variables were analyzed by parametric (Student's t-test / chi-square) statistical methods where appropriate. Pathological and cytokine results were analyzed by nonparametric (Mann-Whitney rank sum / Kruskal-Wallis) statistical tests with appropriate Bonferroni corrected post-hoc analyses. Data is expressed as mean +/- standard error or median with interquartile range respectively.

Table 1: Protocol for formalin fixation and paraffin embedding of aortic valve tissue conduit.

Aortic valve conduit placed into cassette.		
Cassette placed into 10% buffered formalin (short-term).		
Cassette placed into 10% buffered formalin located within the fixation machine.		
Timings for the fixation and embedding:		
i.	Formalin 10% Buffered	10 hrs.
ii.	70% Ethanol	4 hrs.
iii.	80% Ethanol	4 hrs.
iv.	95% Ethanol	4 hrs.
v.	100% Ethanol	4 hrs.
vi.	100% Ethanol	4 hrs.
vii.	100% Ethanol	4 hrs.
viii.	100% Ethanol	4 hrs.
ix.	Xylene	2 hrs.
x.	Xylene	2 hrs.
xi.	Wax	2 hrs.
xii.	Wax	2 hrs.
Aortic valve conduit, following fixation, was embedded into wax block.		

Table 2: Protocol for Haematoxylin and Eosin Staining of Paraffin Sections

Reagent	Time	Procedure
Xylene	5 minutes	Deparaffinization
Xylene	5 minutes	Deparaffinization
Absolute Ethanol	2 minutes	Remove Xylene
Absolute Ethanol	2 minutes	Remove Xylene
95% Ethanol	1 minute	Hydration
95% Ethanol	1 minute	Hydration
80 % Ethanol	1 minute	Hydration
Distilled Water	2 minutes	Hydration
Haematoxylin	6 minutes	Nuclear Stain
Tap Water	Run over slides until water appears to run clear	Remove excess dye
Distilled water	2 minutes	To remove tap water
80% Ethanol	1 minute	
Eosin	30 seconds	Cytoplasmic stain
95% Ethanol	1 minute	Differentiation
95% Ethanol	1 minute	Final Differentiation and begin dehydration
Absolute Ethanol	1-2 minutes	Dehydration
Absolute Ethanol	1-2 minutes	Dehydration
Xylene	2 minutes	Clearing
Xylene	5 minutes	Clearing

The slides were cover slipped with Permount ® and let to dry before viewing under the microscope.

Haematoxylin:

Sigma-Aldrich Mayer's haematoxylin solution (MH532-1L)

The Eosin used is:

Sigma-Aldrich Accustain eosin Y solution (HT110332-1L)

Table 3: Immunohistochemistry Protocol

Aortic valve conduit tissue cut on cryostat, slides stored at -20 degree Celsius.
Air dry slides for 5 minutes.
Fix in -20 degree Celsius acetone in a coupling jar for 3 minutes at 4 degree Celsius.
Let slides air dry for 5 minutes. Encircle sections with pap pen. *
Block tissue in 2% FBS/TBS for 20 minutes. *
Add per encircled section 1 drop avidin sol'n (Vector) and incubate 10 minutes. * ⁺
Flip off solution and add per section 1 drop biotin solution (Vector) and incubate 10 minutes. * ⁺
Flip off solution. Add primary antibody (50-75µL per section) and incubate for specified time. *
Flip off antibody and wash 3x, 2 minutes each in 2% FBS/PBS. *
Add secondary antibody and incubate for 30 minutes. *
Make up avidin/biotin complex from the Vector ABC kit, incubate for 30 minutes. * [^]
Flip off secondary antibody and wash 3x for 5 minutes total in 2% FBS/PBS. *
Add the prepared ABC complex and incubate for 30 minutes. * [^]
Flip off and wash 3x for 5 minutes total in 2% FBS/PBS. *
Prepare DAB substrate and mix well. * [#]
Add substrate to section for 2 minutes. * [#]
Stop by washing in dH2O. *
Counterstain sections in Hematoxylin for 5 minutes and wash in dH2O. *
Dehydrate: 1 minute 70% EtOH, 1 minute 95%, EtOH, 1 minute 100% EtOH times three, followed by 5 minutes Xylene times three. *
Coverslip using mounting media. *
Let dry overnight. *

* All of the following steps are performed at room temperature.

⁺ Vector Laboratories Avidin/Biotin Blocking Kit (Catalogue Number SP-2001)

[^] Vector Laboratories VECTASTAIN Elite ABC Kit (Catalogue Number PK-7200)

[#] BD Pharmingen DAB Substrate Kit (Catalogue Number 550880)

Table 4: Antibodies Utilized

Primary				Secondary	
Antibody	Manufacturer	Dilution	Duration and Temperature	Manufacturer	Dilution
CD68	AbD Serotec (Catalogue Number MCA341B)	1:100	2 hours at room temperature	Jackson ImmunoResearch (Catalogue Number 715-006-150)	1:200
CD3	Abcam (Catalogue Number ab5690)	1:50	Overnight at 4 C°	Jackson ImmunoResearch (Catalogue Number 711-005-152)	1:200

Table 5: Pre-operative Donor and Recipient Animal Weights and Donor Operative Times

Donor Weight		Donor Harvest Time	
Fresh Rat Aortic Valve Conduit, n = 34	188.2 +/- 33.6 g	29 min. +/- 0.3 min.	p = 0.03
Glutaraldehyde-Fixed Rat Aortic Valve Conduit, n = 25	166.3 +/- 30.5 g	34 min. +/- 0.2 min.	
Glutaraldehyde-Fixed Guinea Pig Aortic Valve Conduit, n = 81	130.3 +/- 31.1 g	31 min. +/- 0.2 min.	
Recipient Rat, n = 140	228.9 +/- 29.3 g	N/A	

6. RESULTS:

6.1 Valvular Heart Disease (VHD) in Manitoba

To better understand VHD in Manitoba, we queried Manitoba Adult Cardiac Surgery database. The database has been collecting data on patients undergoing surgery in Manitoba since 1994. It has approximately 13,000 patient records. Prior to 2007, the data could only be queried in a limited fashion. Following extensive data extraction and synthesis to aid with this project, the data was reorganized to allow for more robust investigations.

Figure 1: Incidence of Valvular Heart Surgery in Manitoba. There has been a steady increase in the proportion of patients requiring surgical intervention for VHD. As our population ages, and our interventions become more aggressive, this trend will continue. Over the past decade, there has been ~25% relative increase in the numbers of surgeries performed for VHD. Our local data follows a similar trend that is currently being seen internationally.³

Figure 2: Prosthesis Usage. Within Manitoba, there has been steady trend towards the use of bioprosthetic valves. This trend is partly due to the aging population, but also surgeon and patient preference has had an influence. This change in prosthesis usage also follows what has been seen internationally.³

Figure 3: Risk Factor Profile of Patients Undergoing Cardiac Surgery. Over the past decade, there has been a steady increase in the risk factor profile of patients undergoing all forms of cardiac surgery in Manitoba. Traditionally, these factors have been associated with ischemic heart disease. Emerging evidence suggests that these factors also influence VHD and promote premature bioprosthetic valve failure.

6.2 Novel Experimental Techniques

Several novel techniques with respect to surgical design, histology, and cytokine analyses were employed. Only a handful of studies have been performed using small animal *in vivo* methodology to address questions relating to prosthesis durability for VHD.^{40,68-70,118} To address clinical applicability (Figures 4 & 5) and physiologic variability with *in vivo* work (Figures 6 & 7), the following techniques were employed.

Figure 4: Schematic of Donor and Recipient Operations. Following anesthesia, the donor aortic valve (Lewis rat or Hartley guinea pig) was harvested as a conduit with portion of the anterior mitral valve leaflet and ascending aorta (**A**, **B**, **C**). The conduit (either fresh or glutaraldehyde-fixed) was implanted in a reverse fashion as an interposition graft into the infra-renal abdominal aorta of the recipient (Lewis rat). Blood would flow through the infra-renal aorta, through the aortic valve conduit, down to the iliac arteries (**D**).

Figure 5: In-vivo Schematic of Donor and Recipient Operations. The top panel demonstrates a harvested glutaraldehyde-fixed guinea pig aortic valve conduit (AV). The approximate length is 9-10mm. Adjacent to the conduit is the recipient infra-renal abdominal aorta (Ao). As is evident, there is an appreciable size mismatch. Therefore, the donor valve and abdominal aorta must be tailored prior to implantation. The lower panel demonstrated the exposed retroperitoneal abdominal aorta and inferior vena cava (IVC). The aorta has a diameter less than 1mm. Following proximal and distal control of blood flow with vascular clamps, the donor aortic valve conduit is implanted with continuous running 10-0 nylon suture. The clamps are removed, and blood flow is confirmed through the valve conduit.

Figure 6: Aortic Valve Conduit Histology. There is no standardized methodology to review explanted donor aortic valves. Furthermore, little information exists on this topic. After discussion with the two-blinded clinical pathologists, the following methodology was agreed upon as a standardized approach to grading of valve inflammation. After explant (A), the conduit was formalin fixed and paraffin embedded in the transverse axis [versus longitudinal axis] (B). Following sectioning and staining (C), four areas of the conduit were analyzed for inflammation (D): valve leaflet (V), conduit media (M), conduit commissure (C), and conduit adventia (A).

Figure 7: Aortic Valve Conduit Tissue Cytokine Analysis Protocol. Cytokine analysis on aortic valve tissue has not been previously performed. Furthermore, the physiologic immune response varies from recipient to recipient, making direct comparisons invalid without an internal control. Finally, cytokine concentration will vary depending upon quantity of tissue homogenized. As a result, the following protocol was developed. After explant, a portion (~2mm²) of donor aortic valve conduit was set aside for cytokine analysis. As an internal control (reflecting basal immune response), a similar portion of the recipient aortic valve conduit was also harvested. Following tissue homogenization, both protein quantification and cytokine quantification were performed on the donor and recipient samples. The obtained donor and recipient cytokine concentrations (pg/mL) were standardized to their respective protein quantification (mg/mL). The recipient cytokine value was subtracted from the respective donor value resulting in the final concentration. If the recipient cytokine concentration was undetectable, it was given an arbitrary value of zero. If the recipient value was greater than the donor value, the final cytokine concentration was given an arbitrary value of zero.

Ao. Aorta, AV. aortic valve conduit, IVC. Inferior vena cava

6.3 Validation of the Fixation Process

As previously mentioned, bioprosthetic valves must undergo a mandatory fixation process to stabilize the collagen matrix and reduce antigenicity. The primary component to the fixation process is glutaraldehyde. Several changes to bioprosthetic valves following fixation have been noted.¹² Replicating some of these changes (Figures 8 & 9) would confirm that appropriate fixation has taken place and aid in the clinical applicability of the model.

Figure 8: Transmission Electron Microscopy (TEM) of Guinea Pig Aortic Valve. (A)

TEM of a native guinea pig aortic valve commissure demonstrating intact fibroblast [F] and collagen [C]. (3400x magnification) (B) Following glutaraldehyde fixation, the collagen is fixed in a static form (wavy in this sample), and the extracellular matrix has been made “non-viable” as demonstrated by the fibroblasts now having large vacuoles within its cellular makeup. Others have noted these changes in bioprosthetic valves following fixation.¹²

Figure 9: Transmission Electron Microscopy (TEM) of Guinea Pig Aortic Valve Leaflet.

(A) TEM of a native valve demonstrating the three layers of the aortic valve leaflet, fibrosa [Fb], spongiosa [S], and ventricularis [V]. (4600x magnification). (B) Following glutaraldehyde fixation, though there has been collagen cross-linking to improve initial strength, continuity of valve layers (yellow arrows) are disrupted allowing for eventual stress-related fatigue to have a long-term impact.¹² (9600x magnification)

6.4 Surgical Learning Curve

As with any surgical procedure, there is always a learning curve. As we are examining the role of inflammation, surgical stress does play a role. Therefore, only once the technique has been mastered and is reproducible, can those results be used with any confidence. To assess surgical reliability, intra- and post-operative outcomes were assessed. Donor animal weights varied depending on the implant aortic valve conduit (Table 5). There was a difference in harvest times between the various donor animals (Table 5). Though minor, the glutaraldehyde-fixed rat aortic valve took longer to harvest as a result of the delicate tailoring of the tissue prior to fixation. The recipient animal subjects were younger rodents (~6 weeks) and had a similar weight between all the cohorts (Table 5).

Figure 10: Breakdown of Surgeries Performed. In total, 140 surgeries were performed. A number of surgeries were excluded during the initial (pilot study) period while the surgical, echo, and harvesting protocols were finalized. The overall peri-operative mortality was 25%. Anesthetic issues, intra-operative surgical complications, and post-operative complications were the cause of most deaths. The overall numbers of surgeries in the final analysis were divided between H&E histology and frozen section immunohistochemistry.

	Fresh Rat-to-Rat	GF Rat-to-Rat	GF GP-to-Rat	GF GP-to-Rat (Steroid Treatment)	GF GP-to-Rat (Statin Treatment)
Total Surgeries Performed	— n = 34	— n = 25	— n = 39	— n = 16	— n = 26
Number of Surgeries Excluded	— n = 10	— n = 6	— n = 10	— n = 3	— n = 3
Peri-Operative Mortality	— n = 8	— n = 8	— n = 9	— n = 2	— n = 8
Number of Surgeries in Final Analysis	— n = 16	— n = 11	— n = 20	— n = 11	— n = 15

Figure 11: Per-Operative Mortality. There was no difference in overall peri-operative mortality amongst the various groups. Technically, the fresh rat-to-rat implantation was the easiest surgery to perform, and this was the cohort used during the learning phase. The peri-operative mortality declined throughout the study period from 50% for the first 20 surgeries to less than 20% for the last 20 surgeries.

Figure 12: Operative Data. Intuitively, operative time, particularly with an extensive surgery requiring a mid-line laparotomy and a period of ischemia to the lower body, will have an influence on the recipient. Both total operative time and ischemic time were statistically different amongst the cohorts. This is likely due to the presence of a learning curve, as the treatment cohorts were the last groups to be performed. However, operative duration (ischemic duration) once again declined throughout the study period, from ~170 minutes (~100 minutes) during the first twenty surgeries, to ~120 minutes (~70 minutes) for the last twenty surgeries performed.

Values expressed mean +/- standard error

Figure 13: Post-operative Weight Change. Following small animal abdominal surgery, an initial period of weight loss was observed. By the first post-operative week, weight gain began as the animal returns to normalcy (ie. normal day-to-day behavior). On-going weight gain can be used as a surrogate marker for animal recovery and resolution of the surgical stress response. Amongst the cohorts, the steroid treatment group had a blunted weight gain response following surgery. It is hypothesized that this effect was as a result of the catabolic effect on muscle mass (versus fat distribution) as opposed to on-going post-operative stress.

Values expressed mean +/- standard error

6.5 Murine Echocardiography / Ultrasonography

Unlike ischemic heart disease, there are no biomarkers available for patients who have undergone a bioprosthetic valve implantation. Therefore monitoring patients for potential SVD requires performing serial echocardiograms. As the aortic valve conduit is implanted into the abdominal aorta, when imaging the valve conduit, ultrasonography and not echocardiography is the appropriate terminology for this study. The ability and feasibility to perform imaging studies post-valve implantation remains a key translational component that is currently lacking within the various rodent *in vivo* models.

Figure 14: Cardiac Function. As a confirmatory step, murine echocardiography was used to assess cardiac function on a random selection of post-operative subjects. Visual cardiac function was normal. Below are representative samples of preserved left ventricular wall function.

Figure 15: Aortic Valve Conduit Ultrasonography. To confirm patency, a select cohort of recipients underwent abdominal ultrasonography with the high-resolution ultra-sound probe (Vevo 2100, Visual Sonics). Since the recipients needed to undergo general inhaled anesthetic, the conduit was quite small, and imaging in the abdomen can be difficult due to the presence of intestinal gas, the use of this high-resolution modality was limited to viewing the conduit with no quantification of flow. Panel **A** demonstrates an intra-operative view of a patent aortic valve conduit. Panel **B** is a post-operative long-axis ultrasonographic view of the conduit. Panel **C** confirms patency when Doppler color is added to the imaging field. Panel **D** is a short axis view of the conduit with color Doppler.

Ao. Aorta, AV. aortic valve conduit, IVC. Inferior vena cava

**A. Intra-operative
View**

**B. Ultrasonography:
Long-Axis, No Doppler**

**C. Ultrasonography:
Long-Axis, Doppler**

**D. Ultrasonography:
Short-Axis, Doppler**

Figure 16: Change in Aortic Valve Conduit Flow Velocity. Clinically, blood flow velocity can be measured through a bioprosthetic valve. An increase in this flow velocity would indicate possible narrowing, of which calcific SVD can be a cause. It is unknown if similar findings could be assessed with this surgical protocol during the study period. Blood flow velocities were measured on a select number of recipients at the study startpoint (<7 days post-op) and repeated at the study endpoint (21-28 days post-op). An increase indicates narrowing, suggesting SVD. Overall there was no significant difference in start- and endpoint velocities for either cohort. Any change was most likely due to either improvement in surgical technique or variability and limitations with current imaging technology. As a result of these findings, and the significant resources required for murine imaging, this aspect of the project was abandoned.

Study startpoints and study endpoints expressed as means.

6.6 Explanted Aortic Valve Conduit Histology

Histology remains a key component with this type of *in vivo* study design. As previously mentioned, there is no standardized approach to assess both explanted native and bioprosthetic valves. Furthermore, current findings remain qualitative. Performing quantitative analysis on H&E histological samples is difficult. A standardized approach and blinded assessment by an expert are keys to achieving this. To this extent, Figure 6 outlines our approach to addressing these deficiencies within the current literature. Immunohistochemistry was performed to help identify and quantify the infiltrative cell type seen on H&E histology. As tissues were previously glutaraldehyde-fixed, there was significant non-specific background staining associated with immunohistochemistry on previously formalin-fixed paraffin embedded slides used for routine H&E. Therefore a second series of experiments were required where the explanted glutaraldehyde-fixed tissue was placed into cryo-embedding media to allow for immunohistochemistry on frozen sections.

Figure 17: Representative Samples of Explanted Aortic Valve Conduit Histology. Overall the fresh rat-to-rat cohort demonstrated intact leaflet, media, and adventia and was similar to a native aortic valve. The donor GF rat-to-rat cohort demonstrated changes associated with fixation (i.e. media and leaflet thickening possibly as a result of collagen cross-linking) however did not demonstrate a robust recipient immune response. The GF GP-to-rat cohort mimics the clinical scenario of xenotransplantation. There was evidence of increase recipient inflammation towards the valve conduit. Furthermore, some donor valves demonstrated medial destruction (yellow arrow), and increased inflammatory response (i.e. possible macrophage infiltration) associated with the valve leaflet and commissural area (blue arrow). Treatment of the GF GP-to-rat recipient with steroids was associated with an attenuation of this inflammatory response. Treatment with statins however was not associated with a significant decline in the inflammatory response, as seen by inflammatory cells surrounding the adventia and valve leaflet (green arrows).

A. adventia, C. commissure, M. media, L. leaflet

Fresh Rat-to-Rat

GF Rat-to-Rat

GF GP-to-Rat

GF GP-to-Rat (Steroid Treatment)

GF GP-to-Rat (Statin Treatment)

200x Magnification

Figure 18: Explanted Aortic Valve Conduit Histology. Following blinded assessment by two pathologists, the following quantitative results were obtained. Only a mild-to-moderate recipient inflammatory response was observed. The fresh rat-to-rat cohort had a minimal response while the GF GP-to-rat had a greater response. Steroids administration, but not statins, was associated with attenuation within the recipient immune response. With appropriate statistical methodology, significant changes were difficult to achieve, and the results remain mostly qualitative.

BLINDED ASSESSMENT BY TWO INDEPENDENT PATHOLOGISTS

0 = NIL + = MILD ++ = MODERATE +++ = SEVERE ++++ = VERY SEVERE

Group	Valve Leaflet Inflammation, median (range)	Adventitia Inflammation, median (range)	Medial Inflammation, median (range)	Commissural Involvement of Inflammatory Cells, n (%)	Calcium Deposition, median (range)	Commissural Involvement of Calcium Deposition, n (%)
Fresh Rat-to-Rat n = 11	0 (0 to +)	0 (0 to +) *	0 (0 to 0)	1 (9.1%)	0 (0 to 0)	0 (0%)
GF Rat-to-Rat n = 11	0 (0 to 0)	0 (0 to +++) *	0 (0 to 0)	1 (9.1%)	0 (0 to ++)	1 (20%)
GF GP-to-Rat n = 15	0 (0 to ++)	0 (0 to ++)	0 (0 to ++)	2 (13.3%)	0 (0 to +++)	2 (40%)
GF GP-to-Rat (Steroid Treatment) n = 5	0 (0 to 0)	0 (0 to 0) *	0 (0 to 0)	1 (20%)	0 (0 to 0)	1 (20%)
GF GP-to-Rat (Statin Treatment) n = 9	0 (0 to ++)	++ (+ to ++)	0 (0 to +)	1 (11.1%)	0 (0 to +)	3 (60%)
p -Value	p < 0.05 Amongst Groups	p < 0.001 Amongst Groups	p = NS Between Groups	p = NS Between Groups	p = NS Between Groups	p = NS Between Groups
Post-Hoc Analysis	No Difference Between Groups	* Where significantly different when compared to GF GP-to-Rat (Statin)				

Figure 19: Representative Samples of Positive and Negative Controls for Immunohistochemistry. Rodent spleen and thymus were used for positive controls for CD68 (macrophages) and CD3 (T-cells) respectively. Spleen red pulp stains positive for CD68 while thymus cortex and medulla stain positive for CD3. Primary antibody omission was used as a negative control. To ensure consistent quantification, each series of stainings was done with a positive control. The positive control was used as the color reference in Photoshop[®] when quantifying staining for the respective series of slides. Furthermore, quantification using Photoshop[®] was done in a blinded manner.

NOTE: Due to unforeseen resource issues, immunohistochemistry of the glutaraldehyde-fixation cohort (GF rat-to-rat) was not performed. This cohort, though important in assessing the recipient immune response to fixed donor tissue, is not crucial in assessing the therapeutic value of statin therapy as it does not represent the clinical setting.

CD 68 (Macrophages) and CD 3 (T-cells) [100x Magnification]

Rat Spleen
Primary Antibody Omission

Rat Spleen
CD 68

Rat Thymus
Primary Antibody Omission

Rat Thymus
CD 3

Figure 20: Representative Samples of Explanted Aortic Valve Conduit Immunohistochemistry. **A.** There was sporadic staining of macrophages within the leaflet, media, and adventia of the surgical control fresh rat-to-rat cohort when compared to the negative control (inset panel). The GF GP-to-rat cohort demonstrated significant macrophage staining particularly within the leaflets and adventia. There was a reduction in staining associated with steroid therapy, but not with statin therapy. **B.** Similar findings were seen with T-cell staining. The surgical control fresh rat-to-rat cohort had minimal staining when compared to the experimental GF GP-to-rat cohort. This immune response was attenuated with steroid, but not statin, therapy.

A. CD 68 (Macrophages) [100x Magnification]

Fresh Rat-to-Rat

GF GP-to-Rat

GF GP-to-Rat (Steroid Treatment)

GF GP-to-Rat (Statin Treatment)

B. CD 3 (T-cells) [100x Magnification]

Fresh Rat-to-Rat

GF GP-to-Rat

GF GP-to-Rat (Steroid Treatment)

GF GP-to-Rat (Statin Treatment)

Figure 21: Quantitative Analysis of CD68 Immunostaining. There was a significant difference amongst cohorts with respect to CD68 (macrophage) immunostaining. The xenogeneic experimental cohort (GF GP-to-rat) demonstrated an increased macrophage response. Steroid treatment, unlike statin treatment, was able to attenuate this response, however was not able to return it back to baseline (i.e. surgical control, fresh rat-to-rat). Due to physiological variability, paired between group post-hoc comparisons did not reach significance in many instances.

** and # and ^ represent significant difference between cohorts following corrected post-hoc analyses*

Figure 22: Quantitative Analysis of CD3 Immunostaining. There were no significant differences amongst cohorts with respect CD3 (T-cell) immunostaining. Only within the adventitia, the xenogeneic experimental cohort (GF GP-to-rat) demonstrated an increased T-cell response, however this did not reach significance in paired post-hoc analysis. Steroid treatment, unlike statin treatment, was somewhat able to attenuate this response, however once again this was not significant.

6.7 Cytokine Analysis

To investigate whether histological changes were associated with a systemic and/or localized recipient immune response, serum and aortic valve conduit tissue cytokine analyses were performed. Pre- and post-operative blood samples, in addition to donor and recipient aortic tissue samples were collected. Samples were processed as outlined in the methodology section.

Figure 23: Serum Cytokine Analysis. As to be expected, there was considerable physiologic variability within groups as evident by the variation within the interquartile range (IQR). There was an increase in the cytokine profile between pre- and post-operative samples; however this did not reach statistical significance. The GF GP-to-rat cohort did not demonstrate an elevated cytokine response to the xenogeneic aortic valve conduit. Chronic treatment with steroid and statin therapy was associated with a reduction in serum cytokine levels to below pre-operative basal levels. After appropriate statistical methods (Bonferroni post-hoc correction factor), only the statin therapy cohort had a significant reduction when compared to the other various groups.

	PRE-OPERATIVE CONTROL, n = 10; MEDIAN (IQR)	FRESH RAT-TO-RAT, n = 14; MEDIAN (IQR)	GF RAT-TO-RAT, n = 8; MEDIAN (IQR)	GF GP-TO-RAT, n = 11; MEDIAN (IQR)	GF GP-TO-RAT (STEROID TREATMENT), n = 9 MEDIAN (IQR)	GF GP-TO-RAT (STATIN TREATMENT), n = 7; MEDIAN (IQR)	p - Value
CRP	432821.2 pg/mL (327791.9 - 435955.9)	511686.9 pg/mL (407640.0 - 607711.9)	546074.8 pg/mL (414841.1 - 625031.7)	369389.9 pg/mL (322452.6 - 375063.1)	333706.7 pg/mL (321882.7 - 433765.5)	436519.6 pg/mL (329140.0 - 468178.9)	NS
IL-1 ALPHA	1820.1 pg/mL (1547.9 - 2022.2)	2498.7 pg/mL * (1581.9 - 3030.7)	2812.2 pg/mL (2109.5 - 2988.2)	2443.3 pg/mL # (1571.5 - 3869.7)	804.0 pg/mL (726.9 - 3026.5)	678.6 pg/mL ** (365.8 - 704.8)	*.#
IL-1 BETA	8970.1 pg/mL (7955.2 - 13010.8)	17144.7 pg/mL * (5416.9 - 19222.0)	14093.4 pg/mL (10866.4 - 17793.9)	13780.4 pg/mL # (9878.7 - 17484.9)	2215.9 pg/mL (2020.3 - 20273.3)	1806.6 pg/mL ** (973.6 - 2110.4)	*.#
IL-2	65746.8 pg/mL * (49427.6 - 90587.2)	106898.4 pg/mL # (13504.5 - 224063.8)	122986.5 pg/mL ^ (70878.5 - 180542.2)	44195.8 pg/mL ** (8423.9 - 85047.9)	1787.4 pg/mL (1626.4 - 160346.3)	1622.1 pg/mL **.#, ^ (965.9 - 1653.8)	p < 0.003 *.#, ^, **
IL-6	23851.8 pg/mL (22759.3 - 25845.1)	29905.9 pg/mL * (15510.5 - 32868.7)	29987.6 pg/mL # (26192.5 - 34468.8)	28601.2 pg/mL ^ (21726.8 - 148203.6)	3057.3 pg/mL (2845.7 - 32227.6)	2613.6 pg/mL **.#, ^ (1522.8 - 2953.9)	p < 0.003 *.#, ^
IL-10	19590.7 pg/mL (17270.3 - 23665.7)	25817.5 pg/mL * (15524.4 - 29518.3)	24756.4 pg/mL (20751.1 - 36255.0)	18687.2 pg/mL (14388.0 - 24080.8)	2762.5 pg/mL (2497.4 - 40123.4)	2269.7 pg/mL * (1408.5 - 2626.1)	p < 0.003 *.#
IFN-GAMMA	3609.8 pg/mL (3344.2 - 3847.3)	4157.0 pg/mL * (2598.6 - 5661.0)	4193.3 pg/mL (3675.1 - 5289.1)	3844.0 pg/mL # (2902.1 - 57569.8)	832.9 pg/mL (746.2 - 5688.0)	578.0 pg/mL ** (430.9 - 832.4)	p < 0.003 *
TNF-ALPHA	9956.8 pg/mL (8789.5 - 11340.0)	12830.2 pg/mL * (7078.7 - 15419.4)	12588.3 pg/mL (10881.8 - 16169.0)	10774.1 pg/mL (9052.2 - 12241.1)	449.0 pg/mL (394.6 - 18101.8)	267.8 pg/mL * (193.0 - 385.9)	p < 0.003

Figure 24: Donor Aortic Valve Conduit Cytokine Analysis. To assess a localized recipient response to the donor aortic valve conduit, cytokine analysis of the explanted valve was performed. The calculated values were corrected for degree of tissue homogenization and resting basal recipient immune response (see Figure 7). Grey bars are interquartile range and solid circles are median values. **A.** There was a significant difference between the various cohorts with respect to the expression of Il-1 alpha. Both steroid and statin treatment groups had a reduction, however post-hoc paired analyses between the various cohorts did not reach significance. **B.** With respect to Il-1 beta though there was significant difference between the cohorts. Unlike the steroid treatment cohort, the statin therapy cohort did not demonstrate a reduction and in fact was significantly higher than the surgical control (fresh rat-to-rat). **C & D.** There was no significant difference between the cohorts with regard to IL-2 and IL-6 respectively. **E.** Though there was a significant difference in the expression of IFN-gamma, the paired post-hoc analyses of the treatment cohorts did not demonstrate a significant reduction. **F.** The statin therapy cohort had a significant reduction in TNF-alpha expression compared to the glutaraldehyde-fixation cohort (GF rat-to-rat), however not to the experimental cohort (GF GP-to-rat) cohort. The steroid treatment showed a similar pattern, however this did not reach significance.

** and [#] represent significant difference between cohorts following corrected post-hoc analyses*

C. IL-2

D. IL-6

7. DISCUSSION:

7.1 Summary of Findings

By employing an *in vivo*, intravascular rodent model in an attempt to mimic the clinical scenario of bioprosthetic valve implantation, several important findings were noted. Post glutaraldehyde-fixation of the rodent aortic valve conduit undergoes similar changes as observed following fixation of porcine or bovine bioprosthetic valves prior to implantation. The translational importance of imaging will remain a vital aspect to answering questions related to valvular heart disease in the rodent model. At this time, particularly with small animal imaging, technology is limited to quantifying myocardial function and not valvular function. As seen with explanted human bioprosthetic valves, there is a recipient immune response to the xenogeneic donor aortic valve conduit following implantation. This immune response can somewhat be attenuated with daily steroid treatment. However, daily treatment with HMG-CoA reductase inhibitors (statins) with their proposed anti-inflammatory effects was not able to alter this response. Finally, as seen with clinical research, physiologic variability with *in vivo* models continues to be present a challenge in obtaining meaningful results with regards to complex pathologies.

7.2 Current Clinical Literature

There remains a paucity of literature evaluating medical therapies to attenuate bioprosthetic SVD. Only therapies with a low side-effect profile over a prolonged period

of time would be safe to evaluate. Though anti-inflammatory agents such as steroids might be beneficial, their significant side-effect profile prohibits their use. As a result there has been a growing interest in statins for their pleiotropic anti-inflammatory properties.⁸¹⁻⁸⁴ To date, there have only been two retrospective studies addressing the issue of statin therapy and bioprosthetic SVD.^{25,95}

Antonini-Canterin *et.al.* examined serial echocardiograms on 167 patients following bioprosthetic valve implantation. Among them, 22 were on statins during the follow-up period. Mean follow-up duration was 46 ± 38 months. Their findings were that “... *patients treated with statins had significantly lower rates of bioprosthetic degeneration, as assessed by different parameters*”. Conversely, Kulik *et.al.* performed a much larger retrospective study looking at the role of a variety of lipid lowering agents, including statins. They followed 1,193 patients after bioprosthetic valve implantation, of which 150 were on lipid lowering therapy post surgery. Their mean echocardiographic follow-up period was ~40 months. They found no association between lipid lowering therapy and the progression of SVD.

As previously mentioned, insight into bioprosthetic SVD has been led by the findings associated with native aortic valve stenosis. There have been several large randomized trials examining the role of statins and aortic valve stenosis.⁹¹⁻⁹⁴ Of the four studies, three were negative and did not support the use of statins to slow the progression of aortic stenosis. Despite this, there remain several post-hoc analyses further elucidating the relationship between statins and calcific aortic stenosis.¹¹⁹⁻¹²¹

Several limitations to the above studies, including timing of intervention, dosage of drug, disease burden, and co-morbidities, have been identified as possible reasons for failure to show a difference with statin therapy. These are factors that can be very challenging to address in human clinical studies.

7.3 The Need for a Translational Model

The study of bioprosthetic valve failure specifically, presents a unique challenge to researchers. Within the clinical setting, time to failure and patient clinical heterogeneity introduce variables that prevent any definitive descriptive conclusions. Additionally, with the multiple mechanisms of failure proposed and without a standard classification system, objective findings remain debatable as to causal versus association driven. Finally, as prosthesis development remains largely driven by private industry, information on potential mechanisms of failure remain proprietary.

Though several *in vitro* and *in vivo* models to study bioprosthetic valve failure exist, the restrictions of the methodology limit the applicability of the conclusions.¹²² Changes relating to the fixation process, physiological factors, mechanical stresses, and recipient humoral responses, cannot accurately be duplicated with *in vitro* methodology. Current *in vivo* techniques focus around the analysis of two primary animal subjects; the rodent subcutaneous model and the juvenile ovine model.^{103,123-126} The rodent model involves the implantation of valvular tissue within the subcutaneous space of the recipient. Though

feasible and cost effective, this non-physiologic (i.e. non-intravascular) model is static and does not incorporate recipient blood flow contact; these factors are known to be associated with bioprosthetic valve failure.¹⁰³ The orthotopic ovine model involves the replacement of the native valve with a prosthesis, as performed within the clinical setting. Though physiologic, this model remains very expensive, time consuming with longer study period to observe changes, and finally, is limited to small sample sizes.^{104,122} Therefore, utilizing a rodent model that employs an intravascular aortic xenograft conduit, we have a physiologic, reproducible, and comprehensive *in vivo* model.

7.4 Histological Analysis of Native and Prosthetic Valvular Degeneration

Properly conducted quantitative histological analyses of native and prosthetic valvular disease remains deficient within the literature. Most studies evaluating explanted bioprosthetic valves continue to be qualitative and descriptive.^{71,127,128} Unlike cardiac transplant literature, there are no proper guidelines or classification schemes to aid in the cause and / or degree of failure leading to explantation of bioprosthetic heart valves.¹²⁹ Furthermore, studies that have employed similar *in vivo* rodent models have varied in their approach to quantifying their histological findings and their specific protocols remain unclear.^{40,68-70} To ensure integrity, our protocol followed a very conservative approach.

Firstly, two clinical pathologists reviewed H&E slides of normal native rodent aortic valves. After which, a grading scheme was developed (Section 5.3.1) to help quantify

their future findings. After reviewing the experimental slides individually in a blinded fashion, a consensus was come to for the final grade. It is important that clinical pathologists review slides, as they are very familiar with changes that result from routine sectioning and normal variants that may be overcalled if other non-pathologists were to review them. Furthermore, having a grading scheme developed *a priori*, reduces interpreter bias. By employing this conservative approach, only a minority of donor valves demonstrated an interpretable cellular inflammatory response (Figure 18).

Quantification of immunohistochemistry remains even more challenging, particularly if differences are subtle. We employed a variation on quantification techniques as described by others.^{40,130} Once again a conservative approach to reduce bias was developed. Several photomicrographs of sections for each donor aortic valve conduit were taken using similar microscopy settings. Pictures were blinded to the interpreter. Control positive staining was used as a reference color range. Quantification of background and positive staining was performed on the various photomicrographs. Final results were averaged for each valve conduit. As can be seen from Figures 20, 21, and 22, there remains variability, and only significant changes in cellular infiltration can be commented on.

Though developing a unified grading scheme would be the gold standard, it is possible that physiologic variability and resource limitations may preclude this. Additionally, grading an explanted valve is of only academic use, as it does not influence patient

outcomes; unlike tailoring anti-rejection therapies for heart transplant patients following their biopsies.

7.5 Cytokine Analyses

Cytokines are small proteins (8 – 40,000 daltons) that are involved in various immune cellular processes including proliferation, migration, and communication.¹³¹⁻¹³³ Also known as interleukins, interferons, chemokines, and classically as lymphokines, they are important to both innate and adaptive immunity.¹³³⁻¹³⁶ Cytokines are both pro- and anti-inflammatory, thereby controlling and limiting the deleterious effect of the immune response.¹³⁷ Cytokines exhibit three basic properties as outlined by Tayal and Kalra.¹³³ First is pleiotropy, in that a single cytokine can act on many different types of cells. Second is redundancy, where a similar effect can be initiated by different cytokines. Finally cytokines are “multifunctional”, whereby the same cytokine can regulate different immune functions. For these reasons investigating the cytokine response following implantation may give an insight into the recipient’s immune response.

By using the Luminex ® multi-plex bead-based assay system, we were able to analyze several cytokines from a single small sample at one time. Traditionally this was performed using an enzyme-linked immunosorbent assay (ELISA), which required multiple samples and multiple runs. We chose a kit that had both pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-alpha, and IFN-gamma) and anti-inflammatory cytokines (IL-10).¹³³ IL-1 is released by macrophages, and targets both B-cells and T-cells

promoting proliferation and differentiation. IL-2 is released by T-cells and targets B-cells promoting activation and proliferation. IL-6 released from macrophages promotes antibody production of B-cells. TNF-alpha and IFN-gamma are both released from T-cells and are involved with macrophage activation, chemotaxis, and phagocytosis. IL-10, an anti-inflammatory cytokine, is released from T-cells and inhibits cytokine production and cell function.

Though appealing, studying a recipient's cytokine profile is not without difficulty. Due to their short half-life and their inherent properties as aforementioned, obtaining a clear mechanistic overview remains difficult.^{133,138} This is particularly true with *in vivo* studies where a recipient's response cannot be controlled as with *in vitro* methods, in addition to there being physiological variability with the model. Therefore it is imperative that the results obtained need to be put into context with the histological findings; that being the investigated target cells (i.e. macrophages and T-cells).

7.6 Study Objective #1: To build upon and master a biologically valid novel *in vivo* intravascular rodent model of bioprosthetic valve implantation.

The goal of this objective was to ensure surgical reproducibility. It has been previously shown that surgical stress is related to invasiveness, to duration of surgery, to blood loss, and to post-operative pain.¹³⁹⁻¹⁴¹ It can be hypothesized that these findings would also hold true for small animal surgeries. The *in vivo* rodent model employed was new to our institution and laboratory. As a result, a number of operations were performed prior to

achieving competency both with surgical technique and post-operative care. Of the 140 surgeries performed, 32 (~22%) needed to be excluded. As our study period was 28-days, it is unknown if the post-operative surgical stress associated with this learning curve would have influenced our results. Figures 11 and 12 demonstrate improved performance over time with a significant reduction in mortality and lower limb ischemic and operative duration. Figure 13 demonstrates reasonable return to normalcy and resolution of the surgical stress response as demonstrated by similar post-operative weight gain as compared to control animals.

7.7 Study Objective #2. To ascertain the recipient inflammatory response to valve implantation using histological (hematoxylin & eosin and immunohistochemistry) staining and cytokine (serum and tissue) analyses.

It is well known that the fixation process leads to a “non-viable” ECM.¹⁴² As a result, on-going repair that occurs with native valves is not possible. Furthermore, the “fixed” tissue, though providing strength, does not allow for on-going remodeling leading to cumulative stress damage. Electron microscopy performed on our aortic valve conduits prior to implantation demonstrated an aspect of these changes, including static fixation of collagen and a disturbed ECM. (Figure 8 & 9) It can be hypothesized that following these changes, though making the valve inert with respect to antigenicity, physiologically still elicit a response.

As with any foreign object implanted into a recipient, there will initially be an innate immune response.¹⁴³ Surgical trauma itself will elicit this response.⁴¹ Figure 18 demonstrates that there is an increased cellular inflammatory response following surgical implantation compared with the surgical control (fresh rat-to-rat). Cellular infiltration was seen in varying degrees within the adventia of the conduit, the media, and the valve leaflets. The greatest response was with the xenogeneic GF GP-to-rat cohort, as to be expected. Our results confirm previous findings.⁴⁰ It is difficult however to make direct comparisons as the methodologies differ in how the H&E data was quantified and because of a longer implantation period for this study (28 vs. 21 days). Paired cohort comparisons with appropriate statistical tests did not reach significance. This most likely relates to physiologic variability and the need for more numbers.

The idea that there is an immune response to valve implantation has recently generated some attention. This has been confirmed within the clinical settings as explanted valves have also demonstrated the presence of inflammatory cells.¹²⁸ Immunohistochemistry (Figure 20) confirms that both macrophages and T-cells are present. This would confirm that both the innate and adaptive immune systems were involved.¹⁴³ Manji *et.al.* also showed that there is a humoral response in addition to a cellular response.⁴⁰ This inflammatory response is not limited to xenogeneic implants. Legare *et.al.* also found similar findings with allografts.⁷⁰ Once again, direct comparisons are difficult with these previous studies as methodologies and study durations varied.

How this inflammatory response leads to eventual calcific SVD remains unclear. It is known that unlike native valves, implanted prostheses are unable to tolerate this immune response. Does this immune response follow a similar pathway as seen with native aortic valve calcification? The inflammatory response with native aortic calcification results in the release of pro-inflammatory cytokines, which drive remodeling of the ECM, and in particular promoting valvular interstitial cells (VICs) with an osteoblastic phenotype. There was an increase in cytokine response associated with valve implantation (Figure 24). However, donor valves do not have viable VICs. They do however have an altered endothelial lining (i.e. non-viable). As such, it is plausible that the inactive endothelium of the valve prosthesis may induce a recipient “injury” stem cell response, similar to what is observed with vascular endothelial injury.¹⁴⁴

There is growing evidence suggesting that stem cells play an important role in the pathogenesis of atherosclerosis.¹⁴⁵ It is now accepted that endothelial dysfunction / injury is the “first-step” in the atherosclerotic process.^{145,146} After which there is oxidized lipid penetration with monocyte adhesion and subsequent conversion to macrophages to engulf these lipids. This results in early foam cell formation with the creation of the initial fatty streak.^{61,78,147-150} Several others have proposed that native aortic valve calcification may also follow a similar pathway.^{142,151} Furthermore, with regards to valvular tissue specifically, emerging data suggests that stem cells are found in higher abundance in calcified sclerotic native aortic valves than in control valves.¹⁵² It can be theorized, that infiltrating stem cells, in addition to participating in the pro-inflammatory atherosclerotic process, differentiate into pro-osteogenic phenotypes that could

potentially participate in calcific bioprosthetic SVD. This may also be the link between traditional atherosclerotic risk factors (i.e. smoking, diabetes, metabolic syndrome) and premature bioprosthetic SVD. Therefore, it is conceivable that the local inflammatory milieu present following valve implantation, leads to the migration and differentiation of recipient stem cells that contribute to atherosclerotic bioprosthetic SVD.

In summary, it is well known that the fixation process and the age of the recipient are important factors leading to premature bioprosthetic SVD. Inflammation (no matter the cause) is now becoming an important pathway that most likely links several theories together.

7.8 Study Objective #3: To determine the efficacy of statin therapy to attenuate the recipient inflammatory response.

To evaluate this objective, a control medication known to reduce the inflammatory response was needed. Methylprednisolone is a synthetic glucocorticoid with significant anti-inflammatory properties. By inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF kappa B), important downstream inflammatory mediators (i.e. cytokines) are inhibited.¹⁵³ As a result both humoral and cell mediated immune responses are blunted.

As previously mentioned, the exact mechanism for statins and their pleiotropic anti-inflammatory effects remains unclear. Statins appear to have a much broader but less

powerful effect than steroids. Statins have been shown to have positive effects on the endothelium, antioxidant effects, stabilization of atherosclerotic plaque, inhibition of platelets aggregation, in addition to down regulation of cytokines.¹⁵⁴

Histologically (H&E and immunohistochemistry) steroids, but not statins reduced the inflammatory response associated with aortic valve conduit implantation (Figures 18, 20, 21, and 22). How this pathological finding relates to the recipient cytokine profile remains unclear. Systemically, statins were able to reduce the cytokine profile, even more so than steroids (Figure 23). Tissue cytokine analyses demonstrated a mixed response with statins (Figure 24). Conversely, steroids consistently were able to reduce the localized inflammatory response. This may suggest that there is more of a paracrine effect versus an endocrine effect with respect to cytokine function.

Determining why the difference between steroids and statins remains difficult. It may be that steroids have a broader impact as a result of their mechanism of action (inhibiting intracellular mediators of cytokine production in recipient immune cells) resulting in a decrease in the immune cellular response. It is known that the anti-inflammatory effects of statins are partly mediated through their effects on the endothelium. The donor aortic valve conduit does not have a viable endothelium, therefore it can be hypothesized that an intact endothelium is required. Steroid treatment does not require an intact donor endothelium as it has a systemic effect on the recipient.

However, this would not explain their lack of benefit when used for native aortic valve calcification. In those studies, it was hypothesized that dosage and timing were possible reasons for negative findings. These issues don't apply to our model as recipients were given high-dose statin therapy for the entire study period. It has also been suggested that the anti-inflammatory properties of statins are partially mediated through their effects on cholesterol metabolism.⁹⁴ Our rodent recipients were not hyperlipidemic.

The dosage of statins used also raises some important considerations. Compared to the clinical setting, the dose of rosuvastatin was approximately 10-20 times the maximum dose recommended in human patients.¹⁵⁵ This dose was chosen as it has been shown to have beneficial pleiotropic vascular effects in the rodent model.¹⁰⁸ It should be noted that several commonly used cardiac medications (hydrochlorothiazide, metoprolol, and lisinopril) used in rodent studies have had supra-therapeutic dosages compared to clinical medicine.¹⁰⁸ The need for such high doses is as a result of differences in interspecies metabolism in addition to appropriate species-to-species conversion calculations (i.e. the use of body surface area).^{156,157} However the use of such high doses raises the concern of a recipient hormetic (U-shaped) dose response to statins.¹⁵⁸ As the dosage increases, the pleiotropic effect is lost, and may actually result in a toxic response. There is some evidence to suggest that statins do exhibit a biphasic dose response with respect to endothelial cell migration.¹⁵⁹ Additionally, it is unknown if surrogate markers of statin cytotoxicity (such as creatine kinase) would correlate with this biphasic response. Currently no literature exists with respect to inflammation. Future studies should evaluate this phenomenon with respect to recipient immune response.

In addition to potential dose effect, statins also vary in their biochemical make-up. The family of statins share a common final pathway, that being inhibiting the rate-limiting step of cholesterol biosynthesis (hydroxymethylglutaryl coenzyme A reductase). However, studies have shown that statins can differ in their pleiotropic effects.¹⁶⁰⁻¹⁶⁵ In addition to their various metabolism pathways and derivation methods (fermentation versus synthetic), their hydro- or lipophilic nature also plays an important role.¹⁶⁰⁻¹⁶³ Lipophilic statins (such as atorvastatin), because of their biochemical make-up, non-selectively cross cellular membranes. Within hepatocytes and other cells, they are taken up via passive diffusion. However hydrophilic statins (such as rosuvastatin), require a carrier to be absorbed into hepatocytes. Non-hepatocytes lack this carrier, resulting in hydrophilic statins being hepatocyte selective.^{166,167} The end result being that hydrophilic statins are potentially more efficacious with a lower side-effect profile.^{155,168,169} In our study we used a hydrophilic statin for its novelty within clinical medicine and its efficacy with respect to cholesterol lowering. It can be hypothesized that due to its non-hepatocyte selective nature, the pleiotropic effects of lipophilic statins may be more efficacious on recipient rodent inflammatory cells than hydrophilic statins. To date, there has been no study comparing hydrophilic and lipophilic statins with respect to their anti-inflammatory properties.

Overall our findings are consistent with others that statins have a limited role for patients with VHD and bioprosthetic valves.⁹³⁻⁹⁵ However, the majority of patients will still

require a statin therapy for either hypercholesterolemia or for prevention of ischemic heart disease.

7.9 Future Directions

Electron microscopy remains a powerful tool to assess the ECM of valvular structures. It is unknown if explanted bioprosthetic valves have undergone electron microscopy to assess possible mechanisms for premature failure (i.e. matrix fatigue, calcium mineralization, tissue fluid retention).¹² Examining explanted rodent aortic valve conduits with electron microscopy may reveal novel findings to help aid in the better understanding of premature SVD.

In addition to electron microscopy, donor valve tissue can also be allocated for western-blot analyses and reverse transcription polymerase chain reaction (RT-PCR) analyses. Both techniques are more sensitive than routine histology and may aid in the specific identification and mechanism of the recipient's immune response (i.e. innate versus adaptive).

Furthermore, the use of green fluorescent protein (GFP) transgenic recipient rats is an innovative idea to better understand the immune response following implantation. Not only assessing the degree of recipient response to the donor valve, but also potentially identifying other cell types that may migrate and participate in the calcification process.

We have intentionally utilized a “normal” rat as the recipient to characterize the inflammatory changes that occur with xenotransplantation. As previously mentioned, premature bioprosthetic SVD deterioration is dependent on recipient comorbidities.²⁶⁻²⁸ Employing streptozotocin-induced diabetic, spontaneous hypertensive, and obese Zucker rats as recipient rodents, the impact of diabetes, hypertension, and metabolic syndrome, respectively, on bioprosthetic valve failure could be analyzed. These recipients may in fact benefit from statin therapy as some have suggested.⁹⁴

To further this project’s translational focus, future studies should include explanted human bioprosthetic valves. For this to occur, a dedicated heart valve clinic is required in Manitoba. In addition, with the increasing surgical caseload for VHD, this clinic will be of greater importance. The aim of this heart valve clinic would be to follow VHD patients with serial assessments prior to and post surgical intervention. Other centers have demonstrated the benefit of such a clinic.¹⁷⁰ Bringing together specialized individuals within the field of VHD would promote further bed-to-bench side research and fulfilling the idea of a vertically integrated research program.

7.10 Conclusion

The annual rate of open-heart surgery for patients with valvular heart disease is increasing. Despite decades of research, the ideal synthetic valve replacement prosthesis does not exist. There is growing evidence that bioprosthetic valves once implanted, elicit a host immune response that leads to eventual structural valve deterioration. This project

utilized a novel *in vivo*, intravascular rodent model to test our hypothesis that HMG-CoA reductase inhibitors are able to reduce this recipient immune response. Taken together, our results indicate that HMG-CoA reductase inhibitors are not able to attenuate the inflammatory response associated with valve implantation. Further work is needed to assess if there still remains a role for HMG-CoA reductase inhibitors for this indication.

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