PRECLINICAL BIOCOMPATIBILITY ASSESSMENT OF PEDIATRIC VENTRICULAR ASSIST DEVICES

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A number of heart assist devices including the PediaFlow™ ventricular assist device (VAD), a magnetically levitated mixed flow rotary blood pump, and the Levitronix® PediVAS™, an extracorporeal magnetically levitated centrifugal blood pump are under development to address the urgent need for mechanical circulatory support suitable for children in heart failure. VADs are associated with a host of biological complications including bleeding, thromboembolism, and infection. The biocompatibility of these new devices must be characterized in a preclinical model (juvenile ovines) to ensure their safety and efficacy in children. However, biocompatibility studies in ovines are limited due to a lack of available assays.

Flow cytometric assays were developed to detect ovine platelet activation and function. These assays were applied during in vitro assessment of potential biomimetic coatings for the blood contacting surfaces of pediatric VADs. These assays were then applied in vivo in 5 lambs undergoing a VAD sham surgical procedure for 30 days duration, in 20 lambs implanted with the Levitronix PediVAS for 30 days duration, and in 8 lambs implanted with the three design iterations of the PediaFlow VAD ranging from 6 - 72 days duration. The sham surgical procedure enabled characterization of the effects of the implant surgery on platelet activation.

Bulk phase platelet activation was reduced in blood contacting surfaces that received a biomimetic coating compared to uncoated surfaces which was in agreement with platelet deposition results. Platelet activation levels rose post-operatively in the sham animals and returned to pre-operative levels at approximately two weeks. In PediaFlow and Levitronix implanted animals platelet activation also rose post-operatively and typically returned to baseline levels. In these implants platelet activation consistently rose following pump or animal complications. In a subset of studies platelet activation was elevated for the duration of the study and this high level of activation generally coincided with increased kidney infarcts or thrombus deposition in the cannulae at necropsy.

Overall, the blood biocompatibility of the Levitronix PediVAS and the PediaFlow VAD as represented by a low level of platelet activation observed in the majority of studies is encouraging for the potential clinical use of these devices. The ability of the developed platelet activation assays to differentiate between surface coatings, and to discern trends with respect to pump complications and kidney infarcts following VAD implant demonstrate the utility of the assay in assessing the blood biocompatibility of pediatric heart assist devices.

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PREFACE

First and foremost all glory goes to my Lord and Savior Jesus Christ, my source of strength, my light, my hope, and my salvation. I love you Lord! Without you Lord I would not have made it: I would not have been healed from pneumonia in 1986, and I would not have made it out of the car accident in February 1999. It is by your grace that I am here today! I dedicate this to you.

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1.0 INTRODUCTION

1.1 CONGENITAL HEART DISEASE

36,000 children are born with congenital heart defects in the United States each year. In 2006, congenital heart disease had a total mention mortality of more than 6800 [1]. Some of the congenital heart conditions include tetralogy of fallot, hypoplastic left heart syndrome, transposition of the great arteries, ventricular septal defects, and coarctation of the aorta. Acquired cardiomyopathy also affects these young patients and is the leading cause for listing for heart transplant [2-4]. It has been estimated that approximately 9200 per year require an invasive procedure to repair a life-threatening heart defect within the first year of life. It has been further estimated that at least 20% of the deaths caused by congenital heart disease (CHD) occur due to perioperative ventricular failure, progressive cardiomyopathy or complications following cardiac transplant. These 1,000 to 1,200 pediatric patients represent candidates for sustained mechanical circulatory support [5].

1.2 TREATMENT OF CONGENITAL HEART DISEASE

Treatment of CHD can include medical and surgical intervention as well as non-invasive treatments performed in a cardiac catheterization laboratory. However in the cases of severe refractory heart failure, cardiac transplant and extracorporeal membrane oxygenation (ECMO) are the treatment modalities typically considered. Cardiac transplant is an effective treatment for many of these CHD patients and has 81% 1 year survival and 75% 5 year survival after transplant [6]. However donor organ availability as well as the need to match the size of the donor heart to the recipient contributes to 27% mortality after listing for transplantation for infants and children, which is the highest mortality in solid organ transplantation [7-8]. There are also likely an unknown number of children who could potentially benefit from cardiac transplantation who are never listed for a variety of reasons, including absence of a viable long-term circulatory support modality [5].

ECMO has been a widely used treatment modality for children in cardiac failure for the past 30 years [9-10]. It can also be deployed to provide pulmonary or cardiopulmonary support. Despite its widespread use the duration of support for ECMO is limited to 2-3 weeks and is associated with a litany of complications including bleeding, thrombosis, and infection [11-12]. The likelihood of these complications increases with duration of support which can be problematic as the wait time for a transplant can well exceed several weeks. ECMO use also precludes ambulation, extubation, and rehabilitation, which may negatively impact post transplant survival [5]. The use of ECMO as a bridge to transplant gave patients only a 47-57%

survival rate to receive a transplant [13-15]. More appropriate solutions are needed to chronically support children until their heart recovers or a transplant is made available.

1.3 VENTRICULAR ASSIST DEVICES IN ADULTS AND CHILDREN

Ventricular assist devices (VADs) are mechanical pumps that augment blood flow in patients with failing hearts. In this capacity, VADs are an accepted treatment modality for adults in end-stage heart failure who might otherwise die awaiting transplant [16-17]. VADs were shown to provide a significant survival benefit over optimal medical management in patients ineligible for heart transplant [16]. Despite the success of VADs in adults, development of VAD technology for children has lagged well behind in part because of the much lower number of pediatric patients that would benefit from the technology [11, 18].

In the pediatric heart transplant study (PHTS), VADs were found to be an effective treatment modality in children as a bridge to transplant with 77% of the VAD patients surviving to transplant [19]. Furthermore, it was determined that there was no difference in the 5-yr survival of transplant patients put on VAD when compared to patients who did not require a VAD. In comparison to ECMO had a 47%-57% survival to transplant rate demonstrating VAD support is a marked improvement as a bridge to transplant. While this study shows encouraging results for VAD support for children, the majority of those supported were teenagers [19]. This is because the devices used (Thoratec, Novacor, and HeartMate) were designed for adults and their size limit its use in younger patients. The PHTS also found that the worst outcomes were observed in the youngest patients as well as those that had a diagnosis of congenital heart

disease. It was further observed that less than 50% of infants receive a transplant while listed on the transplant list, while 68% of children ages 1-18 listed were successfully transplanted, underscoring the need for new technology to support infants and young children while awaiting transplantation or until their heart recovers [6, 12]. It has further been observed that the vast majority of children listed for heart transplant and those who die after listing are under 2 years of age [5, 8, 19-20].

The Berlin Heart Excor VAD is a versatile device manufactured in Germany that can support infants and young children; however this device does not yet have Food and Drug Administration (FDA) approval in the United States (US) limiting its availability. The MicroMed Debakey VAD Child has a humanitarian device exemption to be used for children, but its device has approval for children ages 5-16 [21]. To date, there are no VADs with FDA approval available for infants and small children [9, 11]. It is clear that infants would benefit from the increased availability of support options while awaiting heart transplantation [22].

1.4 VENTRICULAR ASSIST DEVICES UNDER DEVELOPMENT FOR CHILDREN

Recognizing the dearth of clinical options for infants and small children in heart failure, the National Institutes of Health Heart and Lung Blood Institute (NHLBI) solicited proposals for development of novel circulatory support systems for infants and children experiencing cardiopulmonary failure and circulatory collapse secondary to congenital and acquired cardiovascular disease [12]. Ultimately, 5 consortiums were funded under the pediatric circulatory support program (PCSP) including an implantable mixed-flow VAD designed

specifically for patients up to 2 years of age (the PediaFlow VAD), another mixed-flow VAD that can be implanted intravascularly or extravascularly depending on patient size (the PediPump), a compact integrated pediatric cardiopulmonary assist systems (the pCAS), an apically implanted axial-flow VAD (the Pediatric Jarvik 2000 Flowmaker), and a pulsatile-flow VAD (the Penn State PVAD). These devices are being developed with the potential to initiate a clinical trial sometime this decade. These devices are generally being indicated for infants from 2 to 25 kg and to provide support up to 6 months. Also under development for pediatric cardiac and cardiopulmonary failure is the Levitronix PediVAS [23]. The PediVAS is an extracorporeal centrifugal pump leveraged from the Levitronix CentriMag, which has been implanted in thousands of patients worldwide [24]. This pump is being designed to provide support for up to 30 days. All together this family of devices is certain to markedly improve the options for small children suffering from heart failure.

1.5 COMPLICATIONS ASSOCIATED WITH VENTRICULAR ASSIST DEVICES

While VADs have provided adults and to a lesser extent, children with cardiac support for their severe heart failure, these devices are associated with a myriad of complications. These complications include bleeding, thromboembolism, and infection [16, 25-38]. These complications can lead to debilitating strokes, sepsis and multi-organ failure [25, 27]. After twenty years of VAD experience these complications while lower in incidence have not been completely eliminated [39]. These biocompatibility limitations are one of the major reasons that VAD technology has not been more widely adopted in adults with less severe heart failure [25, 28].

Use of VAD technology in children and adolescents has unfortunately yielded some of the same complications seen in adults. In the PHTS a 41% infection rate was reported for patients with long term VADs along with a 35% stroke rate for patients implanted with more short term VADs [19]. With the Berlin Heart, a device specifically developed for children, with institutions reporting 20-50% stroke rate, in addition to necessary pump changes due to device thrombosis in children implanted with this device [40-41]. With these complications in mind biocompatibility deserves special consideration in the design of devices specifically aimed for infants and young children.

1.6 CELLULAR ACTIVATION FOLLOWING VENTRICULAR ASSIST DEVICE PLACEMENT

Patients in heart failure typically are in a heightened inflammatory state [31, 42]. Despite this, patients receiving VADs experience a further elevation in cellular activation, cytokines and other humoral factors. Several papers have reported a rise in activated platelets or leukocyte-platelet aggregates following VAD placement [34, 43-46]. Indices of thrombin generation, fibrinolysis, and platelet granule secretion have also been shown to increase following VAD implant [34, 47-48]. Loebe et al reported an elevation of several inflammatory markers including IL-6, TNF-α, and neutrophil elastase following VAD placement. Furthermore, activated T cells have been reported following VAD implantation. In these studies activation of T cells induces T cell death which leads to compensatory B cell hyperactivity. The hyperactivity of B-cells can then result in allosensitization [49]. This overproduction of antibodies can spur

heightened risk of cellular rejection post-transplant and can increase waiting time for cardiac transplant [50-51]. The loss of T cells results in defects in cellular immunity and leads to an immuno-compromised state [26, 52-54]. The implantation of VADs leads to an elevation of many inflammatory, immune and thrombotic factors, and these factors likely play a role in the complications observed in VAD patients.

Also of note in the study of VAD effects on cells is that most new VADs and pediatric VADs under development are rotary devices that possess impellers that spin at high revolutions per minute to generate a positive and continuous flow of blood. These rotary devices expose blood elements to transient high shear. Shear is thought to induce platelet activation and alter leukocyte function with the level of cellular alteration related to the shear magnitude and exposure period to the elevated shear field [55-58]. Overall, the effects of VAD placement, the type of VAD, and VAD configuration on cellular activation and overall biocompatibility are not well understood. Given the myriad of complications and activation of the immune, inflammatory, and coagulation systems following VAD placement, a greater understanding of the effects of VAD placement is merited. Furthermore, additional studies are needed to address the effects of blood-surface interactions that might be unique to the low flow rates necessary to provide cardiac support for pediatric patients [19].

1.7 ANIMAL MODELS IN THE STUDY OF VENTRICULAR ASSIST DEVICES

The use of animals to evaluate cardiovascular devices provides a platform in which to study device effects on cellular activation and biocompatibility. Furthermore, the use of large

animal models to assess VAD performance and basic biocompatibility is essential before proceeding to clinical trials [59-61]. In the past decade circulating activated platelets have been quantified in bovines implanted with VADs previously under development [62-64]. A major advantage in using animals for in vivo studies is that they are typically healthy before device implantation. As stated above this is rarely the case in humans receiving a VAD as they are suffering from cardiac disorders and typically have ongoing inflammation and/or coagulopathies. The relative health of the animal makes it possible to elucidate device effects on cellular activation once the effects of the implantation surgery have dissipated. This has been demonstrated in a study comparing platelet activation between calves receiving a rotary VAD and those receiving a sham surgery. Persistent elevation was noted in the calves implanted with VADs while those calves undergoing a sham surgical procedure had a transient elevation of platelet activation that returned to pre-operative levels within approximately two weeks. Platelet activation associated with the sham surgery could be discounted to show that there was ongoing platelet activation in the VAD-implanted calves. Since the calves were healthy before VAD implantation, ongoing platelet activation could be directly attributed to the device. Furthermore general trends were observed in the level of platelet activation in calves that had an uneventful post-operative course and those that that had thrombotic partial occlusion of the VAD [63]. In another study the employed platelet activation assays were able to distinguish between surface coatings on VADs implanted in calves [62]. Assays investigating leukocyte function and inflammation were also developed for bovines. These assays were measured following VAD implant and showed persistent elevation of leukocyte activation indices after implant [65]. These results demonstrated the utility of using animal models along with robust assays to evaluate VADs preclinically.

Ovines are a common animal model for preclinical cardiovascular device assessment [66-67]. In fact, several groups participating in the NHLBI PCSP have selected the ovine model for in-vivo animal testing of their pediatric VAD [5, 12, 23, 68]. However, tools to assess ovine cellular activation have been historically limited. The only report observed in the literature on ovine platelet activation involved measuring activation in platelet rich plasma, but this study did not evaluate platelet activation following implant of a cardiovascular device [69]. In addition this study did not utilize an assay that specifically assessed activation epitopes expressed on ovine platelets instead looking at enhanced expression of the $GpII_bIII_a$ receptor, a receptor seen on all platelets. Provided the appropriate assays were available they could be used to assess the upregulation of activation markers on ovine platelets providing a snapshot of the level of circulating activated platelets after implant of a cardiovascular device [63-64, 70].

Studies involving leukocyte activation in ovines implanted with cardiovascular devices are even more limited. More specifically there have been no studies to date specifically examining the flow cytometric detection of leukocyte activation epitopes in response to cardiovascular device implantation in ovines. This is in part due to a paucity of commercially available antibodies that are known to recognize ovine leukocytes. However in a recent study a number of commercially available human antibodies were identified that cross-react to ovine leukocyte antigens [71]. These results should aid in furthering the study of ovine leukocytes. Overall, there is a great need for more assay development and additional studies in the ovine model, to understand the effects of novel pediatric VADs and other cardiovascular devices on platelet and leukocyte activation.

1.8 SPECIFIC AIMS

Recently, several pediatric VADs have entered the development stages leading up to potential clinical use in children. The effects of these new generation devices on cellular activation and their effects on the incidence of complications are not understood. The biocompatibility of these new devices must be characterized to ensure their safety and efficacy in children. Preclinical studies involving pediatric VADs are typically conducted in juvenile ovines. However, biocompatibility studies in ovines are limited due to a lack of available assays. In this report ovine platelet and leukocyte activation assays were developed. The platelet activation assays were applied in the in vitro setting and used to assess a covalently attached biomimetic material coating on $TiAl_6V_4$. These assays were also used to quantify platelet activation during in vitro mock circulatory loops containing the PediaFlow and Levitronix PediVAS devices. To quantify the effects of implant surgery on cellular activation, cellular activation was quantified in a series of surgical sham studies. Finally the developed assays were employed to characterize cellular activation temporally in ovines implanted with PediaFlow and Levitronix PediVAS. The results of this work establish novel tools to assess cellular activation in ovines and demonstrate their importance in preclinical development of blood contacting cardiovascular devices. This work further provides information on the suitability of the PediaFlow and Levitronix devices for use in providing cardiac support to aid the thousands of children with failing hearts.

2.0 OVINE PLATELET ACTIVATION AND COAGULATION ASSAY DEVELOPMENT

2.1 FLOW CYTOMETRIC PLATELET ACTIVATION ASSAYS

2.1.1 Introduction

Ovines are a common animal model for preclinical testing of blood-contacting cardiovascular devices including mechanical heart valves, endovascular grafts, and ventricular assist devices (VADs) [66-67, 72-73]. A critical aspect in the design of these devices is the evaluation of their blood biocompatibility. Yet, the biocompatibility data that can be obtained in ovine studies is limited due to a paucity of available assays for evaluating circulating blood elements during the implant period. In particular, there have been no reports in the literature using such techniques to assess temporal platelet activation in ovines implanted with cardiovascular devices.

Flow cytometry permits surface expression of platelet activation-dependent epitopes to be quantified, providing insight on circulating platelets not obtainable with platelet aggregometry and plasma assays for β-thromboglobulin and platelet factor 4 [70]. Circulating activated platelets have been measured in patients with stents, mechanical heart valves, and VADs as well

as in patients suffering from acute myocardial infarction, and ischemic stroke [34, 43, 45, 74-77]. The presence of circulating activated platelets has been suggested as a marker for increased risk of thrombotic complications [74]. Previously several flow cytometric assays to quantify circulating activated bovine platelets and platelet microaggregates were developed and applied to assess circulating activated platelets in calves implanted with rotary VADs [62-64]. The application of similar assays in ovines could yield a greater understanding of device effects on ovine platelet activation during preclinical testing when design changes might be made at a time of reduced regulatory and economic burden.

The objective of this chapter was to develop and characterize flow cytometric platelet assays that could ultimately be applied in the evaluation of cardiovascular devices undergoing in vivo testing in ovines. Specifically we assessed antibodies and Annexin V protein that recognize activated human and bovine platelets in an effort to identify cross-reactive markers that could selectively bind to activated ovine platelets. The identification and characterization of these markers using in vitro stimulation of ovine blood with several platelet agonists is reported.

2.1.2 Methods

Blood collection

Eleven Dorset-cross and Cheviot sheep (3 adult and 8 juvenile) were used in this study. Whole blood was collected from healthy ovines by jugular venipuncture using an 18 gauge 1 ½″ needle with syringe, discarding the first 3 mL. Blood (2.7 mL) was immediately added to tubes containing 0.3 mL of 0.106 M trisodium citrate (Sarstedt, Newton, NC). All experiments were performed at room temperature and blood was added to test tubes within 2 hours of collection.

Evaluation of monoclonal antibodies to detect ovine platelet activation

Blood (5 μL) was transferred from tubes into 12 x 75 mm polystyrene tubes with each of the monoclonal antibodies listed in **Table 2-1**, 5 μL of 20 mM GPRP (Glycine-Proline-Arginine-Proline for inhibition of fibrin polymerization; Anaspec, San Jose, CA) in phosphate buffered saline (PBS; BD Biosciences, San Jose, CA), and 5 μL of goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen, Carlsbad, CA) that was twice the concentration of the primary antibody. Tyrode's buffer (Electron Microscopy Services, Hatfield, PA) with 1% bovine serum albumin (BSA) was added to each tube for a total volume of 50 μL for control samples. A range of concentrations or volumes were evaluated for each antibody. Antibodies that cross-reacted to ovine platelets were optimized to obtain a concentration that efficiently labeled platelets. Optimal concentrations are listed in **Table 2-1**. Antibodies that did not cross-react with ovine platelets are listed in **Table 2-1** with the manufacturer's suggested concentration or antibody volume.

Table 2-1: List of antibodies evaluated

Antibody	Antigen Target	Isotype	Concentration	Volume	Source
Monoclonal antibodies					
PAC-1 Fluorescein	activated human $GPII_bIII_a$	IgM	25 μg/mL	20 μL	BD Biosciences (San Jose, CA)
Anti-human CD109-PE	human platelet actuation factor	IgG_1	20 μg/mL	10 μL	Chemicon (Temecula, CA)
Anti-human CD62P-PE clone # AK-4	human CD62P	IgG_1	*	20 μL	BD Biosciences
Anti-human CD63-Fluorescein	human CD63	IgG_1	*	20 μL	BD Biosciences
BAQ 125A	bovine activated platelet epitope	IgG_1	15 μg/mL	5 μL	Washington State University Monoclonal Antibody Center (WSUMAC; Pullman, WA)
BAQ 56A	bovine activated platelet epitope	IgG_1	15 μg/mL	5 μL	WSUMAC
GC5A	bovine activated platelet epitope	IgG_1	15 μg/mL	5 μL	WSUMAC
Anti-bovine CD63	bovine CD63	IgG_1	*	10 μL	Serotec (Raleigh, NC)
CAPP2A	ovine CD41/61	IgG_1	7.5 μg/mL	5 μL	Veterinary Medical Research & Development (VMRD; Pullman, WA)
GB20A	85 kD protein on bovine platelets	IgG_1	7.5 μg/mL	5 μL	VMRD
GB84A	bovine CD42d	IgG_1	7.5 μg/mL	5 μL	WSUMAC
NPL44-10	human CD62P	IgG_1	25 μg/mL	5 μL	Takara Bio (Shiga, Japan)
MCA2419	human CD62P	IgG_1	25 μg/mL	5 μL	Serotec
MCA2420	human CD62P	IgG_1	25 μg/mL	5 μL	Serotec
6F3	ovine CD62P	IgG_1	25 μg/mL	5 μL	Harvard University (Cambridge, MA)
Isotype control antibodies					
IgM-Fluorescein	IgM isotype control	IgM	25 μg/mL	20 μL	Southern Biotech (Birmingham, AL)
Coli S69	IgG_1 isotype control	IgG_1	matched for each IgG_1 Ab experiment		WSUMAC
rabbit IgG	rabbit IgG isotype control	IgG	30 μg/mL	5 μL	Southern Biotech
Polyclonal antibody					
rabbit polyclonal anti-human CD62P	human CD62P	n/a	30 μg/mL	5 μL	BD Biosciences

* Concentrations not provided by manufacturer

Activated samples were prepared as above, but with 5 μL less of Tyrode's buffer with BSA and with 5 μL of agonist added for a final concentration of either 20 μM adenosine diphosphate (ADP; EMD Biosciences, San Diego, CA), 68 μM thrombin receptor activating peptide-6 (TRAP; Bachem Biosciences, King of Prussia, PA), or 10 μM platelet activating factor (PAF; EMD Biosciences). These agonists were prepared in PBS. Quiescent control and activated samples were incubated for 20 min in the dark with occasional gentle mixing. After incubation, samples were washed with 1 mL of Tyrode's buffer containing 1% BSA and 0.106 M sodium citrate and centrifuged at 132 x g for 10 min. Supernatant was then removed and the pellet was resuspended.

CAPP2A is an antibody that recognizes $GpII_bIII_a$ (CD41/61), an antigen on the surface of resting ovine platelets [69, 78-79]. N-Hydroxysulfosuccinimide-LC-LC-biotin (LC refers to a hydrocarbon chain extender to reduce steric hindrance; Pierce, Rockford, IL) was added to CAPP2A in 20 molar excess to produce CAPP2A-biotin for use as a platelet marker. CAPP2A-biotin (5 μL at 7.5 μg/mL) and streptavidin-phycoerythrin (PE) (5 μL at 73 μg/mL; BD Biosciences) were then added to the samples and incubated and washed as before. After resuspension of the pellet, samples were fixed with 500 μL of 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS. Flow cytometric analysis occurred within approximately 2 h of fixation.

Single platelet scattering events (3500 total) were identified using CAPP2A related PE fluorescence and forward scatter (FSC) from each sample (Figure 2-1A) and assessed for fluorescence at a second wavelength corresponding to the binding of antibodies from **Table 2-1**, using a FACScan flow cytometer (Becton Dickinson [BD], Franklin Lakes, NJ). As shown in Figure 2-2, a standard flow cytometric technique was employed to define activated platelets - a fluorescence intensity threshold mark was set so that 2 ± 0.2% of the single platelet events had fluorescence intensities falling above the mark due to binding of the isotype control antibody.

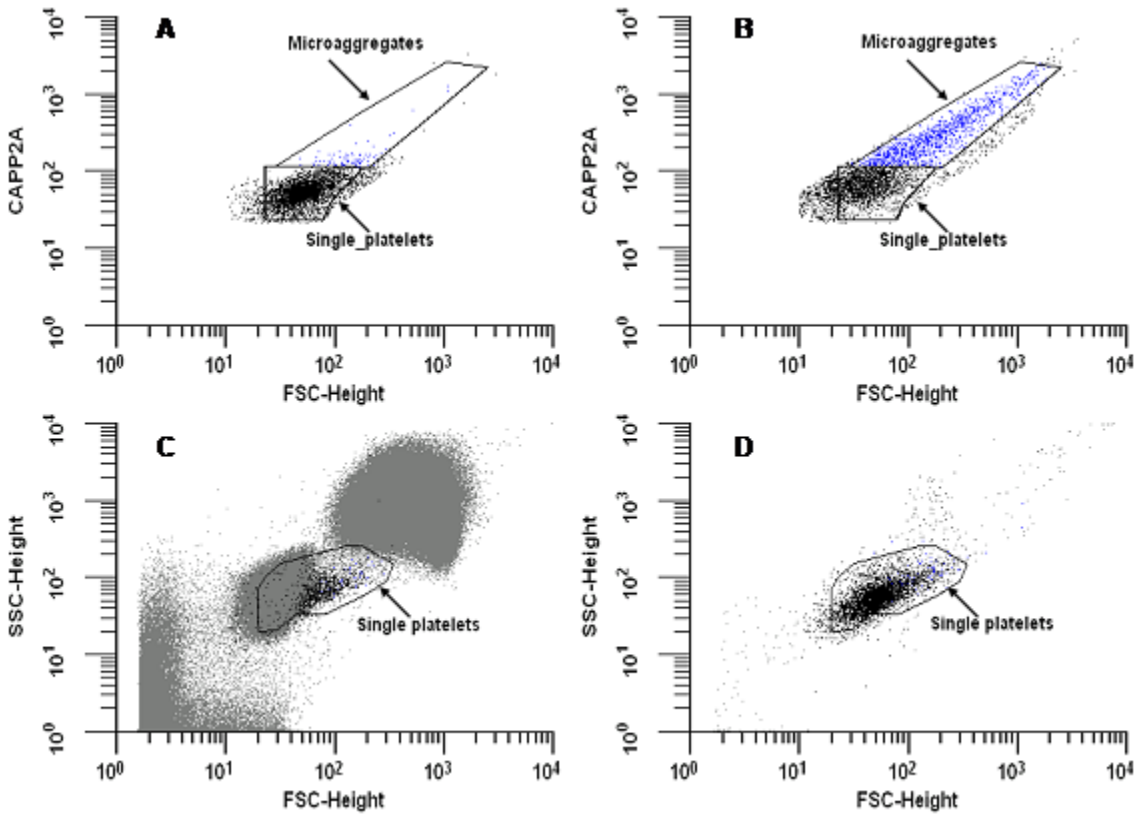

Figure 2-1: Flow cytometric forward scatter plots

CAPP2A binding versus forward scatter for individual scattering events is displayed identifying ovine platelets for an unstimulated sample. (B) Scatter plot of ovine platelets gating with CAPP2A fluorescence and forward scatter for a 10 μM PAF-stimulated sample. Microaggregates bind more CAPP2A and have a higher forward scatter than single platelets. (C) Forward scatter versus side scatter plot for ovine blood cells. The region labeled "single platelets" is where platelets typically reside on such a plot. (D) Forward scatter versus side scatter plot of ovine platelets identified with CAPP2A. There are a number of cells in the single platelet region of panel C that are not in the same region in panel D, suggesting that these cells are not positive for CAPP2A and should not be considered platelets.

Fluorescence associated with each antibody for control and activated samples was compared with respect to this threshold mark (**Figure 2-2B-D**) and reported as a percentage of platelets positive for binding the antibody of interest. Coli S69A (Washington State University Monoclonal Antibody Center [WSUMAC], Pullman, WA) was used as the isotype control for IgG_1 antibodies and goat anti-mouse IgM-Fluorescein (Southern Biotech, Birmingham, AL) served as the isotype control for PAC-1-Fluorescein. Percentage of platelets positive for Coli S69 was 2.1% for control (**Figure 2-2A**) and 5.7% for PAF-stimulated blood (**Figure 2-2B**). Percentage of platelets positive for CD62P as detected by MCA2419 was 11.6% for control (**Figure 2-2C**) and 70.7% for the PAF-stimulated sample (**Figure 2-2D**). Since intermittent clotting of ovine blood was noted during several of the initial antibody binding experiments, 20 mM GPRP was added to the samples to prevent fibrin polymerization. Evaluated antibodies in **Table 2-1** that were conjugated to PE were prepared as above substituting streptavidin-PE with streptavidin-fluorescein (5 μL at 100 μg/mL; BD Biosciences). Samples evaluating the CAPP2A, GB84A, and GB20A antibodies were prepared without the addition of a platelet marker, eliminating the second incubation and wash steps. In these experiments, 5000 total platelet scattering events were collected by forward scatter and side scatter (SSC) in the single platelet region shown in **Figure 2-1C-D**.

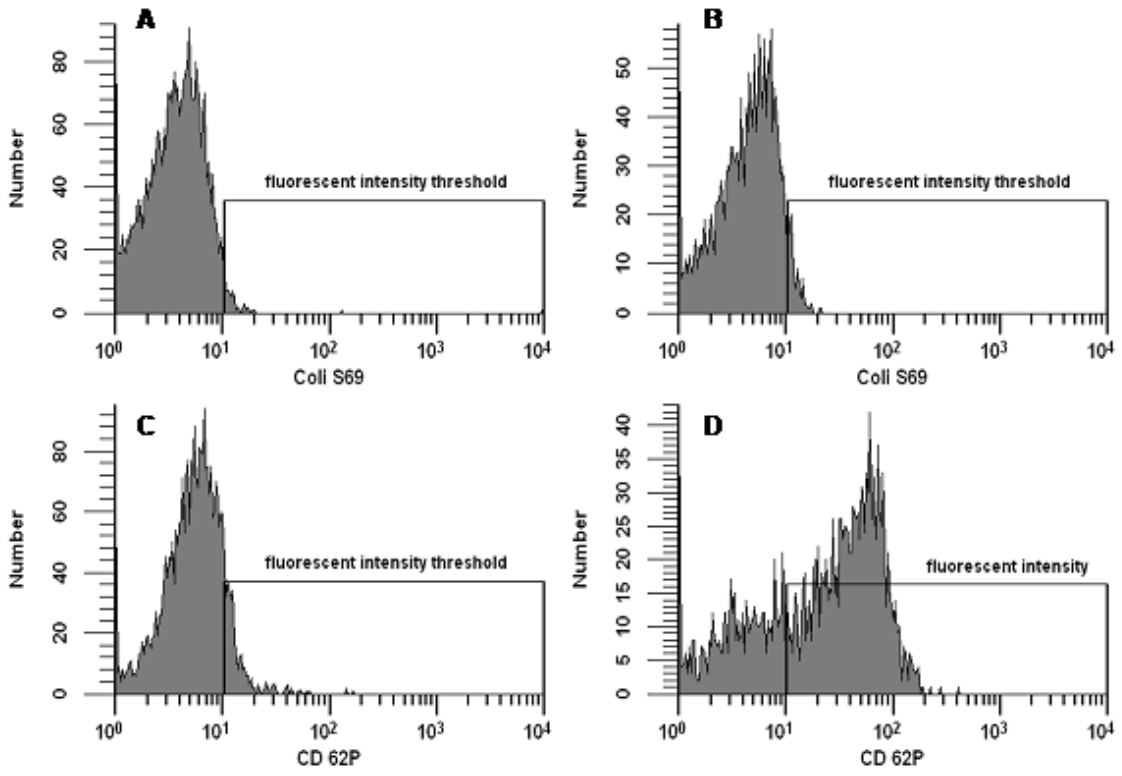

Figure 2-2: Flow cytometric analysis plots

The y axes represent the number of platelets having a fluorescence intensity given on the x-axis (arbitrary units) due to fluorescent antibody binding. The fluorescence intensity of platelets bound with isotype control antibody (Coli S69A) varies little between quiescent control (A) and 10μM PAF (B) stimulated blood. The fluorescence intensity with monoclonal anti-human CD62P antibody (MCA2419) binding for unstimulated (C) and 10μM PAF (D) stimulated ovine platelets shows a marked increase with stimulation.

Measurement of ovine platelet microaggregates

The percentage of platelet-containing microaggregates for samples evaluating NPL44-10, MCA2419, and MCA2420 antibodies was measured. Single platelets were identified with CAPP2A and forward scatter in control samples. Microaggregates were classified as scattering events that bound additional CAPP2A and had higher forward scatter than single platelets (compare **Figure 2-1A and B)**. The percentage of microaggregates was defined as the percent of

microaggregate events relative to the combined number of single platelet and microaggregate events. Microaggregate percents were compared for quiescent control and agonist-stimulated samples. In **Figure 2-1A** (quiescent control sample) the percentage of microaggregates is 2.7 % and in **Figure 2-1B** (PAF-stimulated sample) the microaggregate percentage is 39%.

Evaluation of polyclonal anti-human CD62P Ab to detect ovine platelet activation

Blood (5 μL) was incubated for 20 min with 20 μL of Tyrode's buffer with 1% BSA, 5 μL of 20 mM GPRP, 5 μL of polyclonal rabbit anti-human CD62P antibody (30 μg/mL; BD Biosciences, San Jose, CA) or 5 μL of rabbit IgG (30 μg/mL; Southern Biotech), 5 μL of goat anti-rabbit IgG1-A488 (30 μg/mL; Invitrogen), 5 μL of GB20A (7.5 μg/mL) and 5 μL of goat anti-mouse-IgG1-PE (30 μg/mL; Invitrogen) for quiescent control samples. GB20A binds to an 85 kD protein on ruminant platelets and was used as a platelet marker [79]. Activated samples were prepared similarly to the quiescent control samples using 15 μL of Tyrode's buffer with 1% BSA and 5 μL of agonist for a final concentration of 20 μM ADP or 10 μM PAF.

Samples were incubated and washed as above. Supernatant was removed, the pellet was resuspended, and the sample fixed. Flow cytometric data acquisition was performed within approximately 2 h of fixation. Five thousand single platelets identified by forward scatter and GB20A binding were collected for flow cytometric analysis as above using rabbit IgG as the isotype control antibody.

Evaluation of Annexin V-Fluorescein

Blood (diluted 1:10 in PBS; 20 μL) was added to 265 μL of Annexin V binding buffer (BD Biosciences) for quiescent control samples with 5 μL of Annexin V-Fluorescein (BD Biosciences), 5 μL of 7.5 μg/mL GB20A, and 5 μL of 30 μg/mL of goat anti-mouse IgG-PE for 20 min. Activated samples were prepared as above using 235 μL of Annexin V binding buffer along with 30 μL of one of the following agonists for a final concentration of 20 μM ADP, 10 μM PAF, 5 μM ionomycin (EMD Biosciences), or 5 μM calcium ionophore A23187 (EMD Biosciences). Ionomycin and A23187 were initially dissolved in dimethyl sulfoxide and then diluted in PBS to desired concentration. Flow cytometric analysis occurred within approximately 1 h of blood addition. Five thousand platelets positive for GB20A were assessed for Annexin V binding. The fluorescence intensity threshold mark was set to include the upper 2% of the fluorescence from the Annexin V quiescent control samples.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were performed using SPSS 12.0.1 (SPSS, Chicago, IL). Comparison of means for quiescent and activated samples was calculated using one-way repeated measures ANOVA with the F statistic and Bonferroni post-hoc test. Correlations between NPL44-10, MCA2419, or MCA2420 binding and microaggregate percentage were performed using the Pearson correlation. Significance was considered to exist for $p < 0.05$.

2.1.3 Results

Evaluation of antibodies to detect ovine platelet activation

Monoclonal antibodies against human CD62P (clone# AK-4), human CD63, human CD109, human activated $GPII_bIII_a$ (PAC-1) and anti-bovine CD 63 all demonstrated very low binding to quiescent control samples and did not demonstrate a significant increase in binding to ovine platelets stimulated with 20 μM ADP. Monoclonal antibodies BAQ125A, BAQ56A, GC5A, and 6F3 bound strongly to resting ovine platelets (> 58%) but did not exhibit a significant increase in binding upon activation with 20 μM ADP. These results are summarized in **Table 2-2.** CAPP2A, GB84A, and GB20A all bound at least 80% of resting platelets, without a resultant increase in binding upon activation with 20 μM ADP or 68 μM TRAP as shown in **Figure 2-3**. The polyclonal anti-human CD62P antibody and monoclonal antibodies NPL44-10, MCA2419 and MCA2420 demonstrated statistically significant increases in binding to ovine platelets stimulated by 20 μM ADP or 10 μM PAF when compared to quiescent platelets as shown in **Figure 2-4**. The antibody concentration or volume corresponding to antibody binding results shown in **Table 2-2** and **Figure 2-3** and **Figure 2-4** are listed in **Table 2-1**.

Table 2-2: Antibody binding to ovine platelets

Anti-human Platelet Monoclonal Antibodies; N = 3				
	Human CD63	Human CD62P (clone # AK-4)	Human CD109	PAC-1 FITC
Quiescent	0.2 ± 0.2	3.3 ± 1.5	0.4 ± 0.5	1.0 ± 0.2
20 μM ADP	0.3 ± 0.4	3.5 ± 1.6	1.0 ± 1	1.5 ± 1.0

Anti-bovine and ovine Platelet Monoclonal Antibodies; N = 3					
	Bovine CD63	BAQ125A	BAQ56A	GC5A	6F3
Quiescent	2.0 ± 0	97 ± 2	58 ± 7	99 ± 1	97 ± 2
20 μM ADP	2.1 ± 0.8	95 ± 3	65 ± 0.3	95 ± 7	94 ± 4

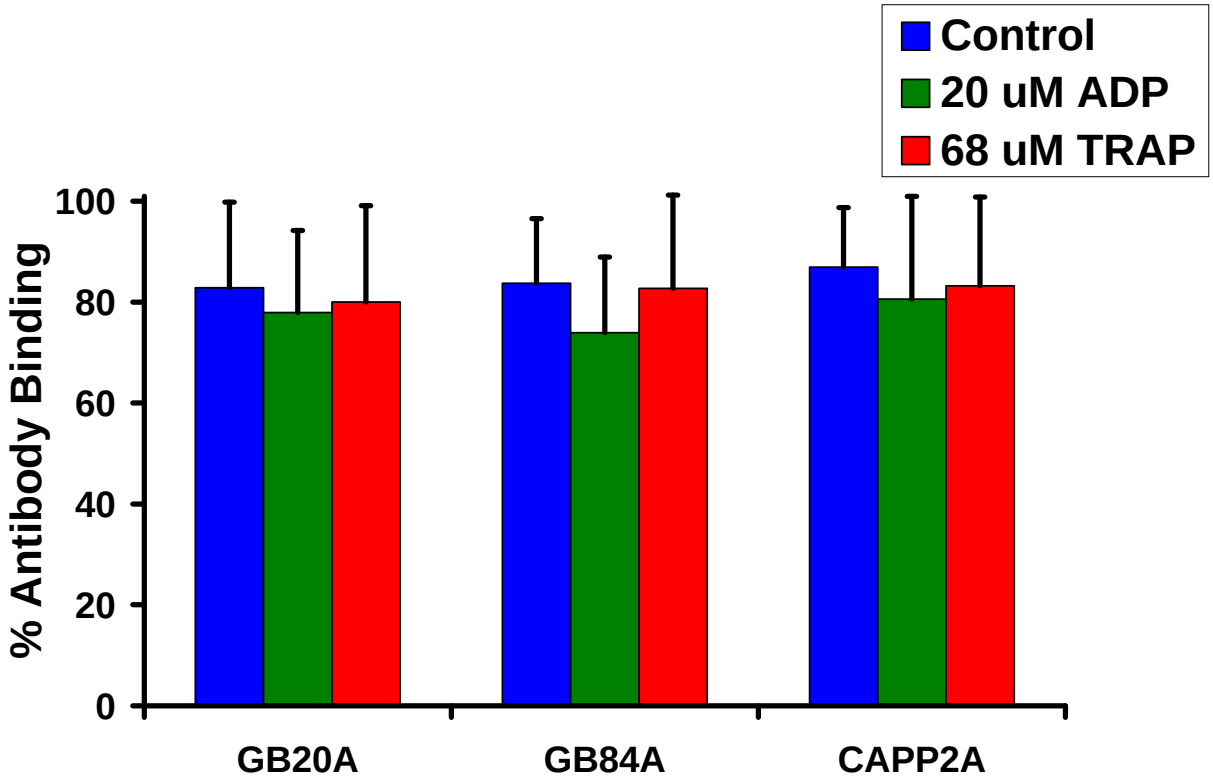

Figure 2-3: GB20A, GB84A, and CAPP2A antibody binding to ovine platelets.

Antibody binding to unstimulated, 20 μM ADP, and 68 μM TRAP-stimulated ovine platelets. N = 7.

Measurement of ovine platelet microaggregates

The percentage of platelet microaggregates increased significantly upon stimulation with 20 μM ADP or 10 μM PAF when compared to quiescent control samples as shown in **Figure 2-5**. There also were significant correlations found between the percentage of platelet microaggregates detected and the binding of the respective monoclonal anti-human CD62P antibodies: MCA2419 ($r = 0.919$, $p < 0.001$) and MCA2420 ($r = 0.908$, $p < 0.001$). The correlation between binding of the NPL44-10 antibody on platelets with percentage of platelet microaggregates was not statistically significant: ($r = 0.304$, $p = 0.149$).

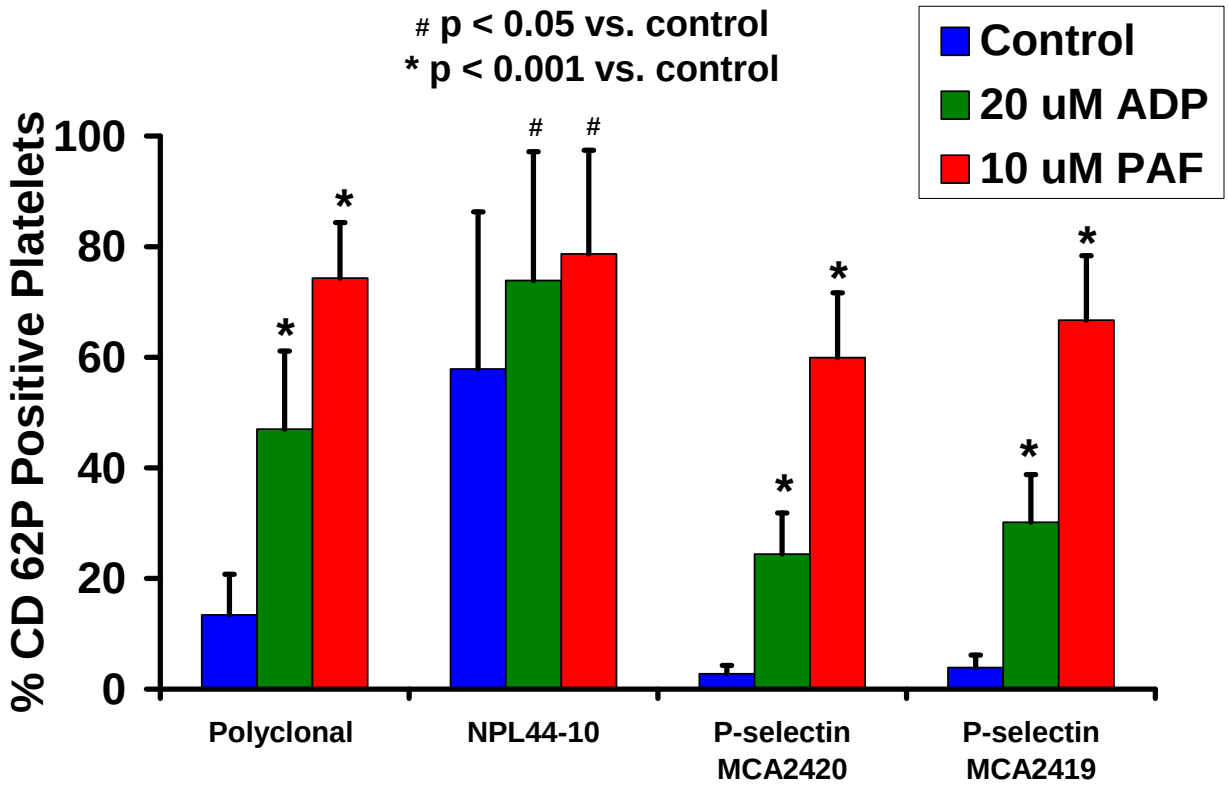

Figure 2-4: CD62P antibody binding to ovine platelets

The percentage of unstimulated and stimulated ovine platelets positive for p-selectin as indicated by the binding of polyclonal and monoclonal anti-human CD62P antibodies. The percentage of CD62P positive

platelets was significantly increased ($p < 0.001$) in 10 μM PAF and 20 μM ADP samples compared to unstimulated controls for the polyclonal CD62P antibody (N = 8), MCA2419 (N = 7) and MCA2420 (N = 7). The percentage of CD62P positive platelets as indicated by NPL44-10 binding was also significantly increased ($p < 0.05$) for samples stimulated with 10 μM PAF (N = 8) and 20 μM ADP (N = 8).

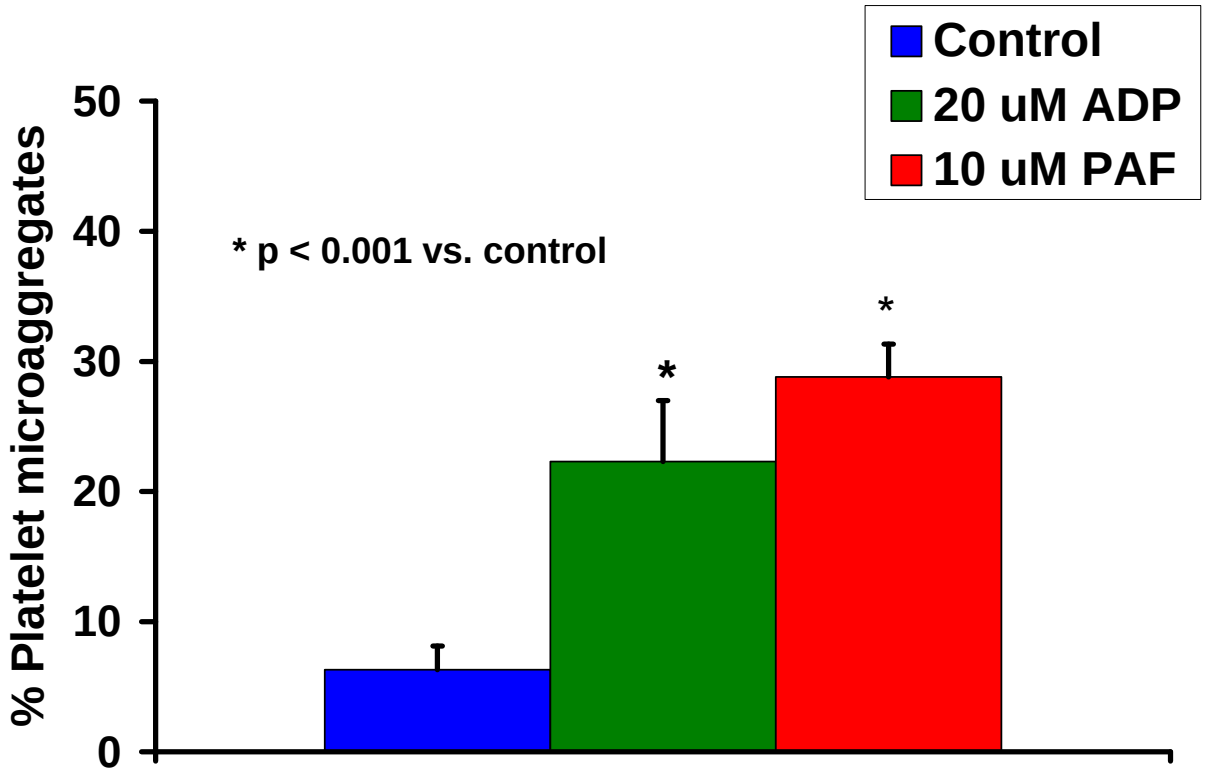

Figure 2-5: Ovine platelet-platelet microaggregates before and after stimulation

The percentage of ovine platelet microaggregates increased significantly with stimulation using 20 μM ADP and 10 μM PAF ($p < 0.001$ versus unstimulated). N = 8 for control and stimulated samples.

Annexin V-Fluorescein Binding

Annexin V exhibited statistically significant binding to ovine platelets stimulated by 5 μM calcium ionophore A23187 and 5 μM ionomycin compared to unstimulated platelets as

seen in **Figure 2-6.** Annexin V binding to 20 μM ADP and 10 μM PAF stimulated samples was not statistically different from control samples.

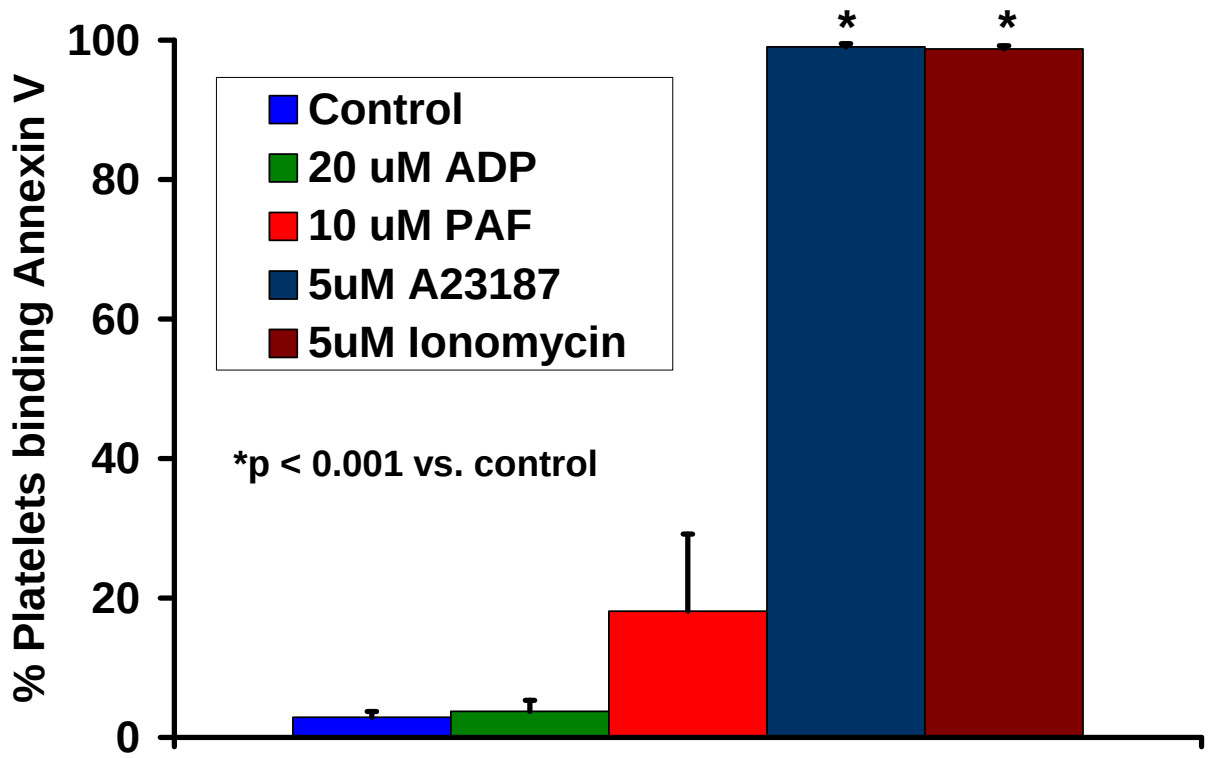

Figure 2-6 Annexin V binding to ovine platelets

Annexin V binding to ovine platelets was significantly increased for samples stimulated with 5 μM ionomycin (N = 9, p < 0.001), and 5 μM calcium ionophore A23187 (N = 9, p < 0.001). Annexin V binding to 20 μM ADP (N = 7) or 10 μM PAF (N = 8), stimulated platelets was not statistically different from binding to control samples.

2.1.4 Discussion

In bovines and humans, identifying platelet events during flow cytometric analysis can be done by simply gating the scattering events using forward and side scatter. However, ovine red

blood cells are smaller than human red blood cells (4.5 versus 8 μm diameter) while platelets are similar in size, making the discrimination of ovine platelets in whole blood by forward and side scatter alone a challenge (**Figure 2-1**) [80-81]. To reliably detect ovine platelets, a platelet specific marker was required. CAPP2A binds to CD41/61 on ovine platelets, and GB84A and GB20A bind to an 85 kD protein on the surface of bovine and ovine platelets [78-79]. The antigen target for GB84A is CD42d, and this is also the presumed antigen target for GB20A, although no confirmation has been reported [82]. Each of these antibodies bound more than 80% of cells (**Figure 2-3**) in the forward scatter vs. side scatter region that contain platelets in quiescent control samples. The percentage of cells that these antibodies bound decreased in ADP-stimulated samples. This is probably indicative of single platelets forming platelet microaggregates that because of their increased size no longer reside in the forward scatter vs. side scatter region typically occupied by single platelets. This further illustrates the need for a platelet marker to discriminate ovine single platelets and ovine platelet microaggregates from other cells. CAPP2A and GB20A were subsequently utilized to label platelets in experiments evaluating the binding of various activation-specific antibodies and Annexin V protein.

The goal of our in vitro characterization experiments was to investigate the ability of candidate antibodies and Annexin V to increase in binding to activated ovine platelets. The stimulation of platelets with agonists that would likely result in secondary generation of thrombin and other agonist release was an inherent feature of the study. To specifically avert fibrin polymerization while allowing thrombin generation, GPRP peptide was utilized. The use of GPRP to prevent fibrin polymerization caused by thrombin has become an accepted practice in assessment of platelet function using flow cytometry in whole blood [83-85]. TRAP, a peptide

fragment of the tethered ligand receptor for thrombin, can directly activate platelets without the generation of a fibrin clot and is an alternative to using GPRP to inhibit the secondary effects of thrombin [70]. However, in our hands TRAP did not induce expression of activation-dependent epitopes on ovine platelets.

While it is of interest to allow thrombin generation following stimulation with agonists in these studies, it would not be desirable to have unanticipated thrombin generation occurring prior to agonist stimulation for assay characterization. Since fibrin formation was sporadically observed in some blood draws (these samples were not utilized), the question arises as to whether there might be artifactual activation due to the presence of thrombin in some samples that did not rise to the level necessary for fibrin formation. Our results suggest that such artifact does not appear to be relevant for the assays that are showing strong sensitivity to platelet activation and that thus might find further use in device evaluation. Specifically, if thrombin were variably present, then the unstimulated control samples for the MCA2419 and MCA2420 assays (**Figure 2-4**) would not show low levels of activation and have low variance. Of note, the values for these assays were not normalized to the control; rather all values including control were normalized to isotype-control antibody binding. Activation due to consistently uncontrolled or variably uncontrolled thrombin would show up in the magnitude and variance of the unactivated control data respectively. Furthermore, if the platelets were activated by thrombin it is unlikely that they would be able to respond to the extent observed with agonist addition.

P-selectin (CD62P) is located within the platelet alpha granule and mediates platelet-neutrophil adhesion. Upon activation the alpha granule fuses with the cell membrane, expressing

p-selectin on the cell surface [70, 86]. 6F3 was the only antibody in this study that was specifically raised against an ovine antigen (p-selectin); however, its very high binding on the resting platelet (**Table 2-2**), precluded its use as an effective marker for ovine platelet activation [87]. The monoclonal antibody against human CD62P (clone # AK-4) showed no cross-reactivity to ovine platelets. The polyclonal anti-human CD62P antibody, NPL44-10, MCA2419 and MCA2420 were also raised against human p-selectin, however these antibodies cross-reacted and selectively bound to activated ovine platelets (**Figure 2-4**). MCA2419 and MCA2420 exhibited the lowest quiescent control binding, and produced the greatest fold increase in relative binding between control and activated samples. The higher control binding with the polyclonal CD62P antibody was not unexpected given that a number of antigens would be targeted. The higher background binding seen with NPL44-10 was less expected. In addition, NPL44-10 demonstrated much higher variation then the other cross-reactive CD62P antibodies. Because of this variation, it seems that NPL44-10 is not the ideal antibody for quantifying p-selectin expression on ovine platelets temporally. For the purposes of one-time in-vitro experiments however, NPL44-10 may still be useful. The differences in cross-reactivity and affinity for the resting platelet between the monoclonal anti-human CD62P antibodies may be due to the different epitopes to which the different antibody clones bound.

Monoclonal antibodies BAQ56A, BAQ125A, and GC5A selectively bound to unknown epitopes on the surface of activated bovine platelets and were successfully applied to assess circulating activated platelets in calves implanted with VADs [63-64]. In ovines these antibodies demonstrated much higher binding to quiescent platelets than was observed with calves and selective binding to activated ovine platelets was not observed. NPL44-10 and the anti-bovine

CD63 antibody also selectively bound to activated bovine platelets [62]. In ovines, NPL44-10 selectively bound to activated ovine platelets, but had high background binding, whereas the anti-CD63 antibody did not cross-react to a meaningful degree.

The percentage of platelet microaggregates increased upon stimulation with PAF and ADP (**Figure 2-5**). Formation of platelet-platelet and possibly platelet-leukocyte microaggregates can follow platelet activation. This phenomenon was noted in our study; a significant correlation was observed between ovine platelets expressing p-selectin (assessed with MCA2419 and MCA2420) and the formation of platelet microaggregates after stimulation with ADP and PAF. Quantification of platelet microaggregates provides an additional index with which to assess platelet activation and, at least in bovines, appears to be a marker of very high levels of in vivo platelet activation [63].

Annexin V binds to negatively charged phospholipids including phosphatidyl serine, which serve as a catalytic surface for coagulation reactions on platelets [88-89]. Annexin V selectively bound to ovine platelets stimulated by the calcium ionophores A23187 and ionomycin (**Figure 2-6**). Calcium ionophores increase intracellular calcium, inducing the translocation of phosphatidyl serine to the platelet surface among other effects. Somewhat surprisingly though, Annexin V did not selectively bind to ADP or PAF-stimulated platelets, suggesting that these agonists did not stimulate the translocation of phosphatidyl serine onto the ovine platelet surface, despite inducing p-selectin expression and microaggregate formation. This might suggest that Annexin V binding to platelets is a less sensitive means to quantify platelet activation when compared to assays that target CD62P expression. Annexin V's response to

ADP-stimulated ovine platelets, was more consistent with the response to ADP-stimulated human platelets, than with ADP- stimulated bovine platelets where Annexin V has been shown to bind preferentially [63, 89].

The use of animals to evaluate cardiovascular devices is an essential part of device development as success in animals can provide some assurance that a device will be safe when used in humans. A drawback to the use of animals for biocompatibility testing is that animals and human platelets respond differently to external stimuli. Goodman reported that sheep and pig platelets adhere and spread differently than human platelets on several common biomaterials [66]. Pelagalli et al also reported differences in the adhesion of animal and human platelets to immobilized fibrinogen [67]. These differences must be considered in interpreting the animal platelet response to artificial organs and extrapolating this information to predict human platelet behavior. It seems likely however that general trends in platelet activation would hold true: design modifications that reduce platelet activation in animals would most likely cause a reduction in human platelet activation.

A major advantage in using animals for in vivo studies is that they are typically healthy before device implantation. This is rarely the case in humans receiving a device who are suffering from a variety of cardiac disorders and typically have ongoing inflammation and coagulopathies. The relative health of the animal makes it possible to elucidate device effects on platelet activation once the effects of the implantation surgery have dissipated.

Flow cytometry provides potentially more sensitive information about device effects on platelets because it can provide some degree of temporal resolution of platelet activation trends in vivo, whereas assessment of platelet deposition on an implanted device and detection of end organ infarcts can only be adequately assessed during necropsy [64]. As mentioned in **Section 1.7,** flow cytometric platelet activation assays were able to discern trends in platelet activation between calves having uneventful VAD implantation periods and calves experiencing partial thrombotic obstruction of the VAD [63]. These assays were also sensitive enough to detect significant differences in the levels of circulating activated platelets for different blood-contacting surface coatings in VAD-implanted calves [62]. Similar experiments in ovines using the assays described in this report could suggest materials or surface coatings that would reduce device-related platelet activation. Experiments evaluating the effects of fluid path, shear stress, and type of blood-contacting materials on platelet activation may help to elucidate underlying contributors to platelet activation observed with artificial organs, and provide insight into potential design modifications that could enhance device biocompatibility before the device is tested in humans.

2.2 NORMAL THROMBOELASTOGRAPH VALUES FOR OVINES

2.2.1 Introduction

The thromboelastograph (TEG, Haemoscope Corp; Niles, IL) is a diagnostic analytical machine that assesses the coagulative state of blood. A blood sample is incubated in the machine at 37°C and is rotated 4°45′ repeatedly clockwise and counterclockwise and assessed for its clot

dynamics. Rotation movement is converted by an electromagnetic transducer to an electrical signal and sent to the computer. The TEG machine can potentially provide more comprehensive information about the coagulative state of blood when compared to partial thromboplastin time, prothrombin time, or other clotting time parameters. While typically used in humans, the TEG could potentially be of benefit in the ovine model, provided normal ovine TEG values were established. The TEG could potentially provide insight on the coagulative state of ovine blood following VAD implant, which could then provide insight into the biocompatibility of a device. The TEG might also provide insight into the level or effectiveness of the anticoagulation strategy being employed during an implant study The TEG has already been deployed as a tool to monitor coagulation in total artificial heart and VAD patients previously [90-92]. Of note, there is also some limited recent data reported on assessment of the ovine response to clopidogrel using the TEG [93]. The objective of this section was to utilize the TEG to establish normal ovine TEG values for use during preclinical pediatric VAD development.

The TEG machine measures a series of values related to the coagulative state of blood that include:

- **R-time**- time to first clot
- **K-time** - time to clot of a certain strength
- **Angle**- rate of clot formation
- **G**- clot firmness
- **MA**- maximum amplitude- measurement of maximum strength of the developed clot. Fibrin contributes to MA however, platelets is the most significant contribution to the clot strength.

- **CI**- Coagulation Index, assessment of overall coagulation status

2.2.2 Methods

Ovine blood (2.7 mL) was drawn via jugular venipuncture or through a jugular venous catheter placed pre-operatively before a VAD implant into a tube containing 0.3 mL of sodium citrate. 20 μL of 0.2 M calcium chloride was added to a TEG sample cup. Blood (340 μL) was then added to the sample cup and the TEG was initiated. A total of 45 sheep were evaluated. Multiple TEG values for a single sheep were averaged together before calculating the final average.

2.2.3 Results

Sheep TEG values (R, K, angle, G, MA, CI) are plotted along with normal human TEG values provided by Haemoscope Corp.

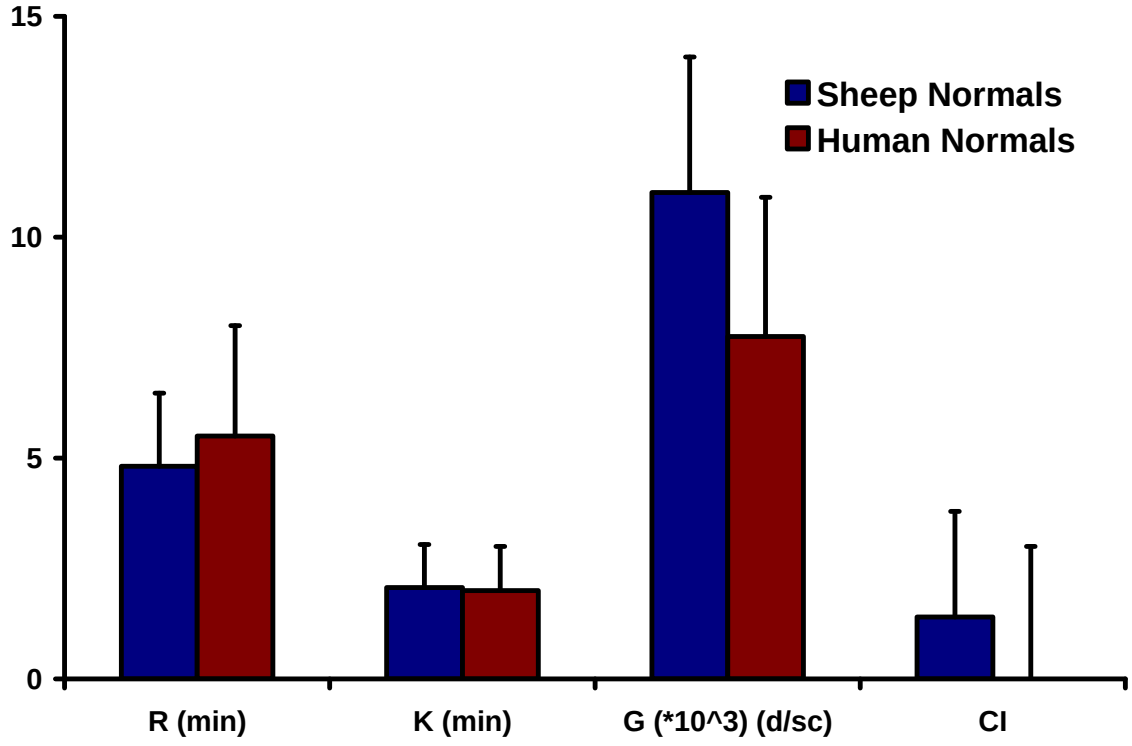

Figure 2-7 Thromboelastograph values (R, K, G, CI) for ovines and humans

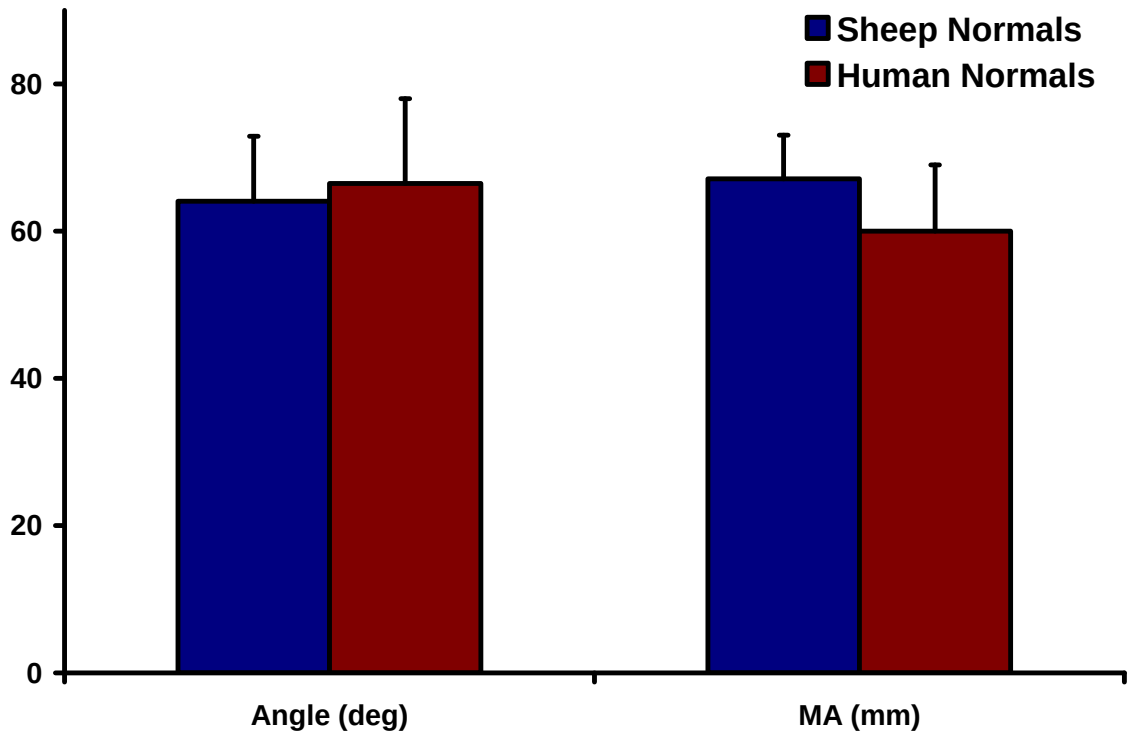

Figure 2-8 Thromboelastograph values (Angle and MA) for ovines and humans

2.2.4 Discussion

Sheep TEG values generally show a higher coagulative state when compared to human blood evidenced by the lower R-time, higher clot firmness, coagulation index and maximum amplitude. The use of the TEG should be useful to assess anticoagulation status and may become a comparison marker to assess the status and function of platelets along with the developed flow cytometric platelet activation assays from **Section 2.1**. Maximum amplitude in particular should hold promise as a potential comparison marker as it computes the platelet contribution to the strength of a clot.

2.3 IN VITRO ASSESSMENT OF COATINGS FOR VENTRICULAR ASSIST DEVICES

2.3.1 Introduction

Blood contacting surfaces in VADs are typically composed of titanium or the titanium alloy, $TiAl_6V_4$. While these surfaces are considered to be somewhat biocompatible thrombosis and thromboembolism are still significant causes of morbidity and mortality in VAD patients [16, 27, 32-33, 37-38]. As a result VAD patients are typically on systemic anticoagulation and or antiplatelet medications to reduce thromboembolic risks. The use of these medications however increases the risk of bleeding in these fragile patients and while the risk of thromboembolic events is diminished, it is not eliminated.

A potential way to reduce the thrombotic risk and the necessity for anticoagulation is to improve the biocompatibility of the blood contacting surface. VAD manufacturers have longed strived to improve this interface [94]. Methacryloyloxyethylphosphorylcholine (MPC)-bearing polymers are phospholipid polymers that mimic the cell membrane that have emerged as a promising candidate for surface coatings. MPC-bearing polymers have been applied to a variety of surfaces including the surfaces on biomedical devices [95-100]. In animal studies, MPC was adhered to the blood contacting surface of the Evaheart pump and compared to the Evaheart pump with a diamond like carbon coating, which is a clinically used coating [100]. In this study, Evaheart pumps coated with MPC had significantly less platelet activation when compared to the DLC coated pumps [62]. The key drawback to the Evaheart pumps coated with MPC is that the MPC was not covalently attached to the surface and was shown to elute over time [100]. In this

section we sought to covalently attach an MPC containing polymer to the $TiAl_6V_4$ surface and sought to assess the biocompatibility of the resultant surface in terms of activated platelets in the bulk blood by flow cytometric assessment.

2.3.2 Methods

The process of covalently attaching a MPC bearing polymer onto the $TiAl_6V_4$ surface (Ti-PMA) is illustrated in the manuscript by Ye et al [101]. The acute in vitro blood biocompatibility of the modified surfaces was evaluated by continuous rocking in a hematology mixer for 2 hrs with anti-coagulated (6 U/mL heparin) ovine blood (**Figure 2-9**). The following surfaces were evaluated: negative control (no surface), polystyrene (positive control), unmodified $TiAl_6V_4$, a silanated $TiAl_6V_4$ (Ti-APS, an intermediate step in production of Ti-PMA), and Ti-PMA. Thrombotic deposition was assessed macroscopically and with scanning electron microscopy. Flow cytometry was used to quantify platelet activation as indicated by Annexin V binding for ovine blood samples after contact with the unmodified and modified titanium samples. Blood samples were prepared for flow cytometric analysis as described in **Section 2.1.2** instead using 250 μL of Annexin V binding buffer and the level of platelet activation was determined as described in **Section 2.1.2**. Activation levels from five independent samples were averaged for each surface type.

Figure 2-9: Rocker setup for in vitro assessment of materials

2.3.3 Results

Scanning electron micrographs of modified titanium surfaces (Ti-PMA) had dramatically lower platelet deposition than unmodified titanium and polystyrene and Ti-APS samples. In concert with the deposition results; platelet activation of blood in contact with the phospholipid modified titanium samples (Ti-PMA) was significantly lower than that measured for the unmodified titanium and polystyrene samples as reported by Ye et al [101].

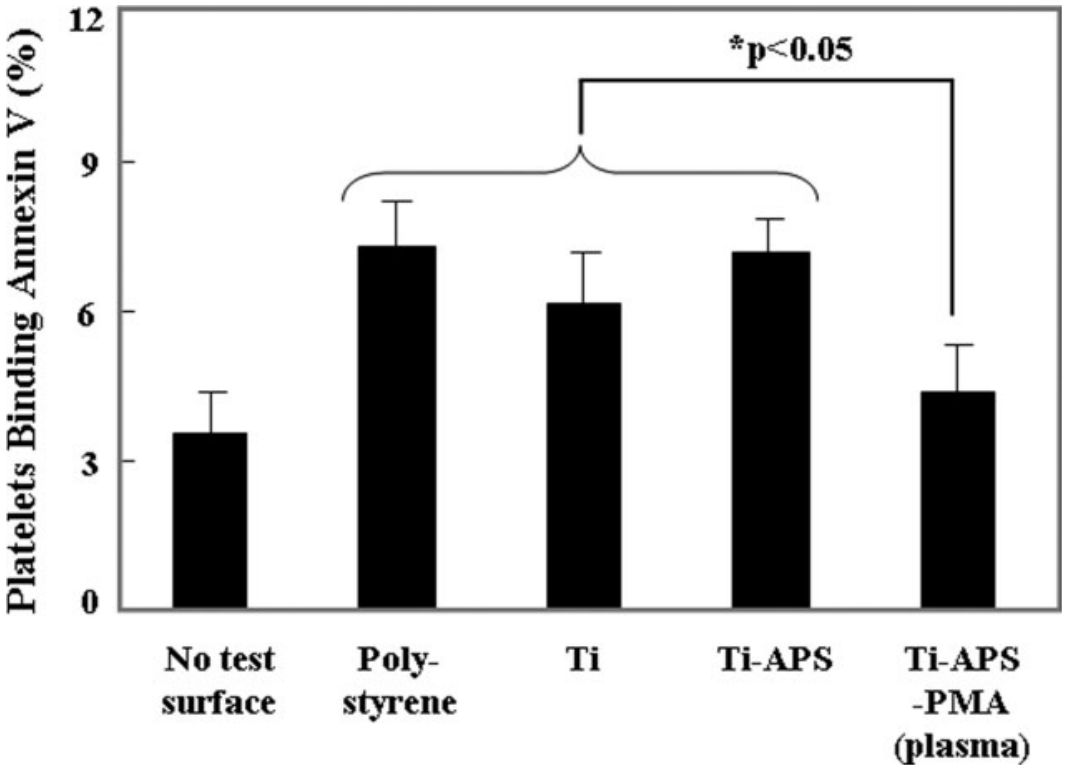

Figure 2-10 Platelet Activation following material contact

Quantification of activated platelets in the bulk phase of ovine blood after surface contact under continuous rocking. No test surface indicates blood from a rocked tube into which no test surface was placed. Platelet activation was quantified by flow cytometric measurement of Annexin V binding.

2.3.4 Discussion

A dramatic reduction in platelet deposition was accompanied by significantly reduced platelet activation results. Ratner asserted that the assessment of blood compatibility by platelet deposition alone is inadequate stating that platelets that do not deposit onto a biomaterial surface can still become activated and circulate in the bulk blood [102]. For example, platelet activation could be used to discriminate between two surfaces where no platelet deposition was present but had different levels of platelet activation in the circulating bulk blood. The platelet deposition

results alone would suggest both surfaces are promising yet the activation results would enable further discriminate between the different surfaces. The results of this section demonstrate a reduction in platelet activation and deposition on the MPC-coated titanium surface. These results also demonstrate that the flow cytometric assays developed in **Section 2.1** proved useful during the in vitro biocompatibility assessment of materials. The use of flow cytometry to detect diminished platelet activation in surfaces passivated with MPC was demonstrated in subsequent studies where MPC was attached onto $TiAl_6V_4$ via a UV-grafting method and through a single-step method onto to $TiAl_6V_4$ [103-104]. The ability to critically discern the level of platelet activation from different blood contacting surfaces illustrates the utility of such assays in the armamentarium of biocompatibility assessment of cardiovascular devices.

2.4 CONCLUSIONS

This chapter summarizes our effort to develop platelet activation and coagulation assays for assessment in ovines. Platelets could not be adequately identified by forward and side scatter alone; therefore a platelet marker was necessary. CAPP2A, GB20A, and GB84A bound to platelets regardless of activation state and can be used for platelet labeling. Several proteins that bound to activated human and bovine platelets cross-reacted and selectively bound to activated ovine platelets with statistical significance. These proteins included: NPL44-10, MCA2419, MCA2420, Annexin V, and a polyclonal anti-human CD62P antibody, and their binding was indicative of surface expression of p-selectin and a procoagulant platelet surface. An assay to detect microaggregates was also developed and the percentage of aggregates was shown to increase upon stimulation. Normal TEG values were established for the ovine model and holds

potential to monitor coagulation and may serve as a complementary platelet assessment tool along with the developed flow cytometric assays. Finally, flow cytometric assays were able to discern between different surfaces in the in vitro setting signifying the utility of the assays as important tools for improving the performance and safety of blood-contacting devices in the ovine model of preclinical testing.

3.0 OVINE LEUKOCYTE ACTIVATION ASSAY DEVELOPMENT

3.1 INTRODUCTION

Infection is a very significant complication observed in patients undergoing VAD placement [16, 35-39]. Although bleeding and thromboembolic complications tend to level off in cumulative incidence after the first month following implant infection incidence continues to rise [36]. The study of leukocyte response following VAD placement is therefore merited. Increased leukocyte-platelet aggregates (LPA) have been observed in patients following strokes [76]. Monocyte-platelet aggregates have also been shown to be elevated in patients with acute myocardial infarction [105]. Monocyte-platelet aggregates also increased after VAD implantation and following coronary stent placement [34, 46, 106]. Granulocyte-platelet aggregates increased after VAD implantation, following stent, and mechanical heart valve placement [34, 36, 75, 106-107]. The presence of these aggregates represents increased thrombotic and inflammatory potential in the peripheral blood and as such would be meaningful measures of biocompatibility following VAD placement in preclinical models. Leukocyte-platelet aggregates have been studied in calves implanted with adult VADs [65]. In these studies leukocyte platelet aggregates were shown to rise sharply following surgery and remained elevated above baseline for the duration of the study period indicating there was ongoing

inflammation. Such assays that could detect these markers in the ovine model would then be attractive given the bovine model.

Patients implanted with ventricular assist devices have activation-induced T-cell death accompanied with B-cell hyperactivity [26, 37, 49, 52-54]. Assays to detect leukocyte activation may be useful in uncovering the mechanisms underlying alterations in leukocyte function observed in VAD patients. CD25 is the IL-2 receptor alpha chain, and CD86 is a marker of B-cell activation [108-109]. With respect to T-cells the IL-2 alpha chain is up-regulated to form a robust IL-2 receptor that can bind to IL-2 with a much higher affinity. Antibodies that could block CD25 have been further speculated to be a potential way to prevent VAD-induced T-cell activation and subsequent B-cell activation [49, 110]. Upon activation CD4 T-cells express MHC class II antigens on their cell surface [111-112]. It has been further noted that the CD4/CD8 ratio has been shown to dramatically decrease in some VAD patients in as early as a month following VAD implantation [52]. The study of how VADs impact leukocytes is therefore merited. We sought to extend the developed flow cytometric assays from **Chapter 2** to include assays to assess leukocyte platelet aggregate formation and lymphocyte activation. In this chapter we sought to develop potential assays to quantify leukocyte activation and then assessed them following in vitro platelet and leukocyte agonist stimulation.

3.2 LEUKOCYTE PLATELET AGGREGATE ASSAY DEVELOPMENT

3.2.1 Methods

To begin characterization of leukocyte-platelet aggregates (LPA) it was necessary to identify antibodies that could label ovine granulocytes and monocytes. An anti-human CD14 antibody (AbD Serotec) was reported to label human monocytes and cross-reacted to bovine and ovine monocytes. This antibody was initially used to label ovine monocytes, however the CAM36A antibody (VMRD) was determined to provide better monocyte labeling as seen in **Figure 3-1**. The PG68A antibody (VMRD) labels ovine granulocytes as seen in **Figure 3-2**.

Ovine whole blood samples were collected via jugular venipuncture and incubated with 20 mM fibrinolysis inhibiting factor (GPRP), agonist and CAM36A or PG68A for 20 or 120 minutes. The following agonists were used (listed at final concentration): 20 μM adenosine diphosphate (ADP, 20 min incubation), 10 μM platelet activating factor (PAF, 20 min incubation), 500 nM and 0.2 μM Phorbol-myristate-acetate (PMA, 120 min incubation) and 0.2 μM Phorbol-myristate-acetate combined with 5 μM calcium ionophore A23187 (120 min incubation). At the end of the incubation period samples were washed with citrated tyrode's buffer. Samples were then incubated with CAPP2A-biotin (**prepared in Chapter 2**) and streptavidin-PE for 20 minutes. Ammonium chloride potassium buffer, 8.29 gm NH_4Cl, 1.0 gm $KHCO_3$, 0.0372 gm disodium ethylenediamine tetra-acetic acid/L distilled H_2O; (ACK buffer) was added to lyse the RBCs and the samples were centrifuged and resuspended as before, washed with Tyrode's buffer with citrate, then fixed with 1% paraformaldehyde. Using flow

cytometry, granulocyte and monocyte cell populations were analyzed to determine if they formed aggregates with platelets (evidenced by binding to CAPP2A) following agonist stimulation. IgG1-biotin (Serotec) was used as the isotype control for the leukocytes binding to CAPP2A. MCA2419 conjugated to Alexa 488 (MCA2419-A488) was used as a positive control to ensure the functionality of the platelet agonists. IgG1-Alexa Fluor 488 was used as the isotype control for MCA2419 binding.

3.2.2 Results

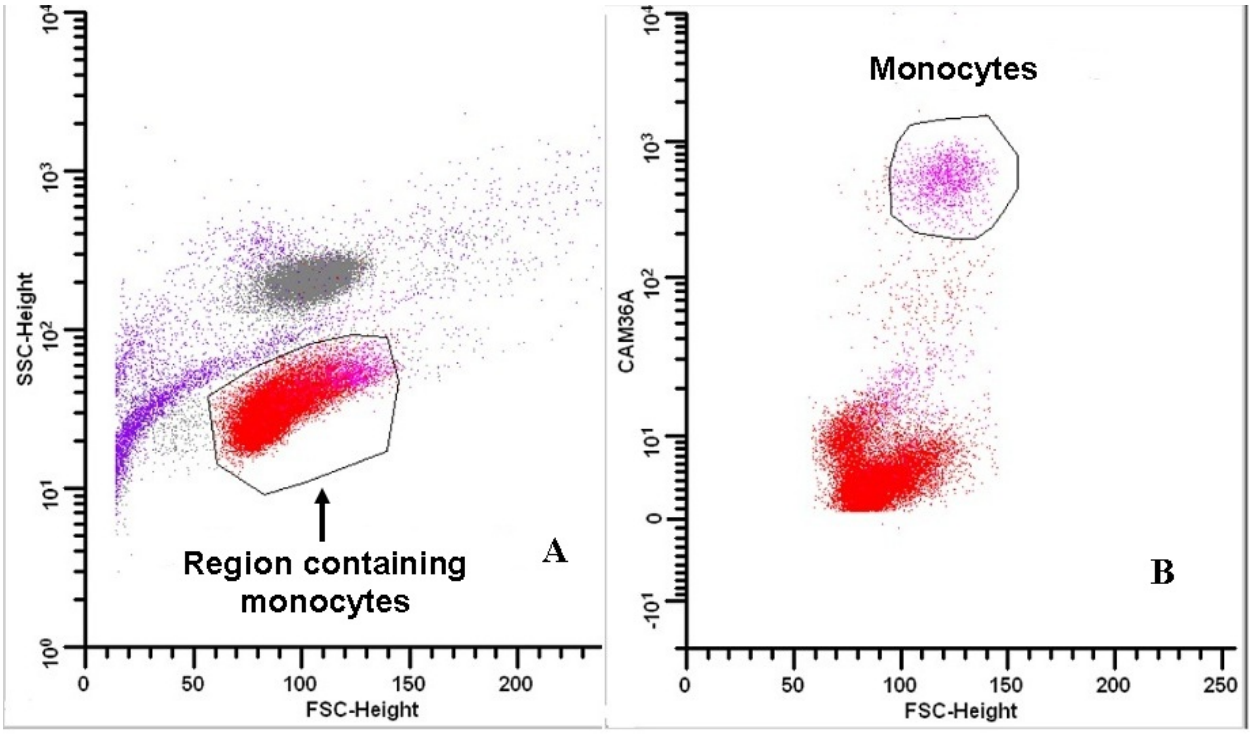

Figure 3-1 Ovine monocyte flow cytometry scatter plots

A) Monocyte region on (FSC) vs. Side Scatter (SSC) B) CAM36A antibody binding vs. Forward Scatter (FSC)

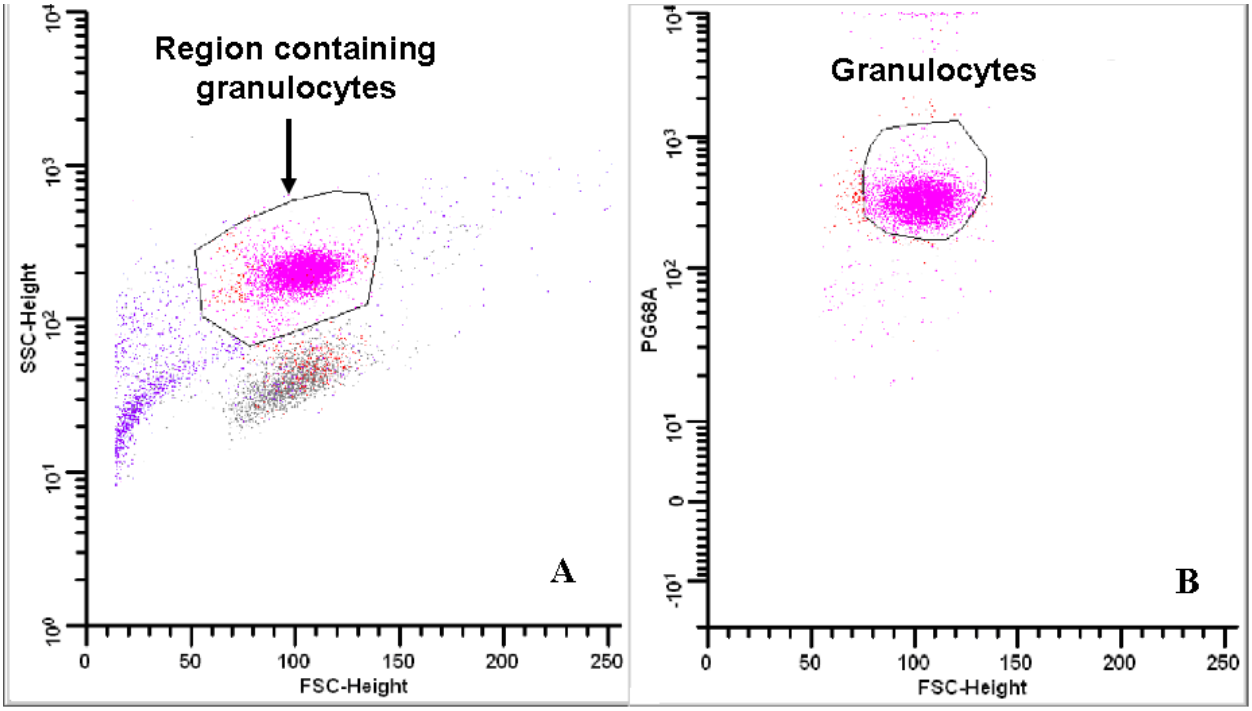

Figure 3-2 Ovine granulocyte flow cytometry scatter plots

A) Granulocyte region on (FSC) vs. Side Scatter (SSC) B) PG68A antibody binding vs. Forward Scatter (FSC) plot

Platelet p-selectin expression was induced by 20 μM adenosine diphosphate (ADP), 10 μM platelet activating factor (PAF), 0.2 μM phorbol-myristate-acetate (PMA), or 1 U/mL thrombin as quantified by MCA2419. P-selectin expression was not induced after incubation with 500 nM or 5μM TRAP (expected). Despite this, granulocyte (GPAs) and monocyte platelet aggregates (MPAs) did not increase after stimulation with ADP, PAF, thrombin, or TRAP as illustrated in **Figure 3-3 and Figure 3-4**.

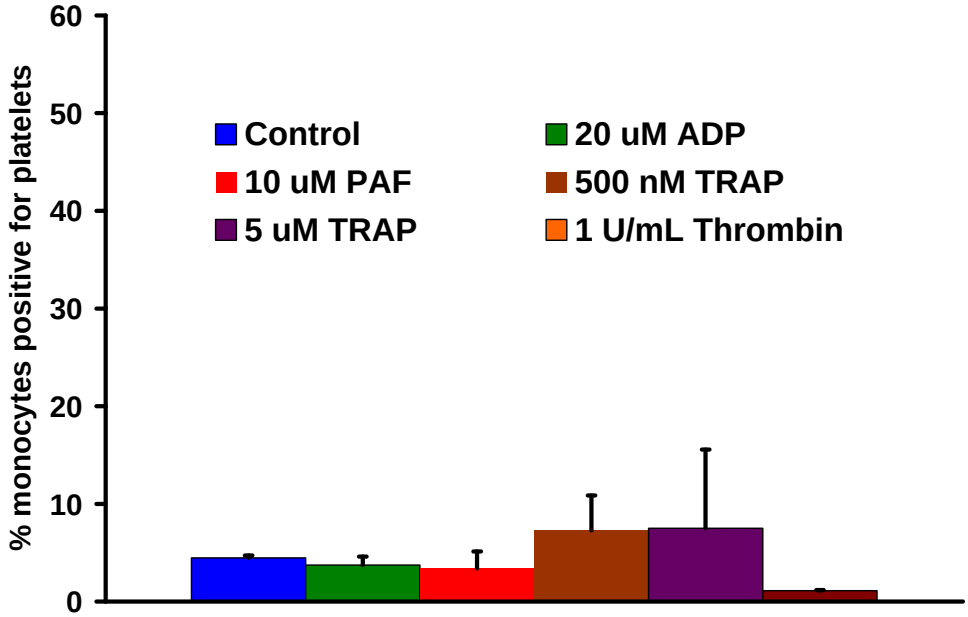

Figure 3-3 Monocyte platelet aggregates following platelet agonist stimulation

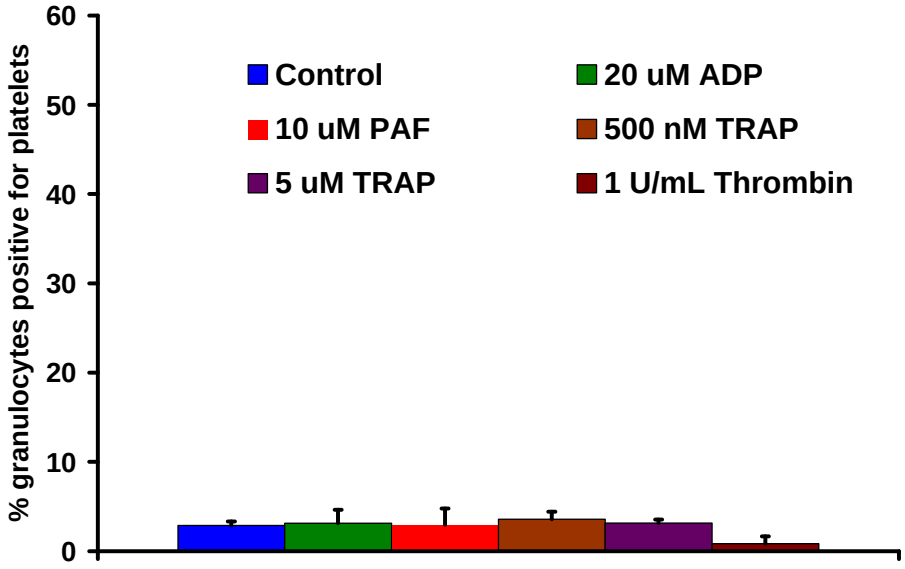

Figure 3-4 Granulocyte platelet aggregates follow platelet agonist stimulation

Monocyte and granulocyte platelet aggregates (**Figure 3-5 and Figure 3-6**) did increase following stimulation with PMA and PMA combined with the calcium ionophore A23187. These

graphs demonstrated that granulocytes and monocytes bind to platelets after stimulation and indicate that leukocyte platelet aggregates could be an indicator of ovine leukocyte activation.

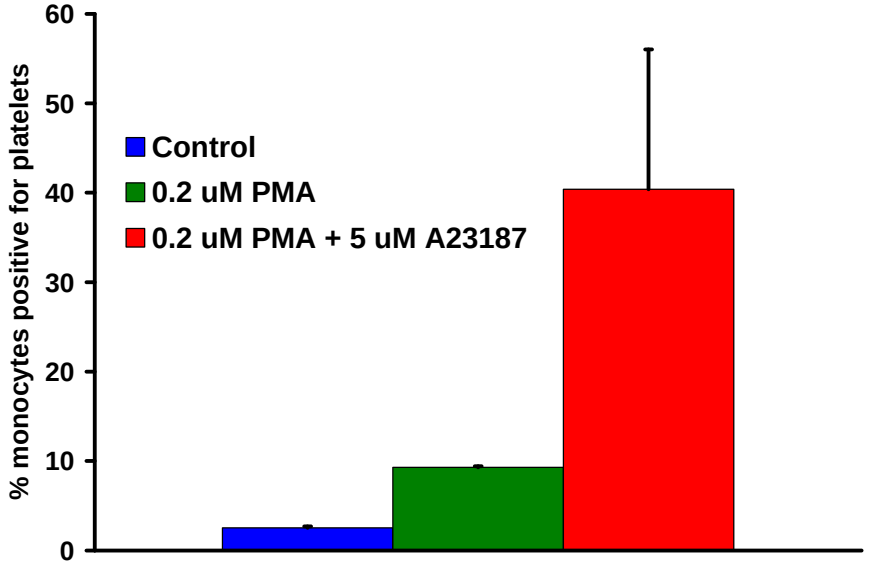

Figure 3-5 Monocyte platelet aggregates following combined leukocyte/platelet agonist stimulation

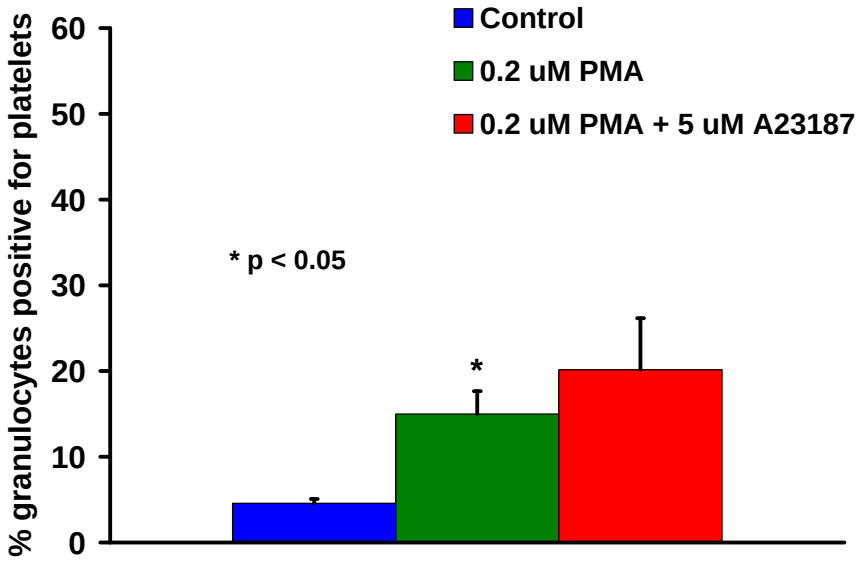

Figure 3-6 Granulocyte platelet aggregates following combined leukocyte/platelet agonist stimulation

3.2.3 Discussion

As observed in **Figure 3-1** and **Figure 3-2** the antibodies PG68A and CAM36A successfully identified granulocytes and monocytes. The results observed in **Figure 3-5 and Figure 3-6** did show some inconsistency in the generation of LPAs following stimulation. This was most notable in **Figure 3-5** where there were large error bars for MPA generation following stimulation with PMA and ionomycin. It was somewhat surprising that TRAP did not induce GPAs or MPAs as this agonist induce leukocyte platelet aggregates in the bovine model [65] It was also interesting to note that platelet agonists, ADP, thrombin, PAF, and TRAP did not induce the formation of LPAs, while PMA and PMA combined with A23187, which would be considered a platelet and leukocyte agonist, did generate LPA formation.. This might indicate that platelet stimulation alone is not enough to form LPAs in ovines; leukocytes must be stimulated for LPA formation to occur. This would further suggest that the presence of increased LPAs in ovines is indicative of an inflammatory response.

Despite some success with the generation of leukocyte platelet aggregates, IgG1 (isotype negative control) binding to leukocytes also increased following stimulation with PMA and PMA combined with calcium ionophore A23187. These results cast doubt about the utility of leukocyte platelet aggregates as a marker of leukocyte activation. It is possible that the IgG1 antibody may be binding to the Fc receptor on leukocytes. Activation of leukocytes induces expression of many markers; the increase in markers on the surface increases the probability of an isotype control antibody binding to leukocytes. This may in fact be the cause of the increased isotype control antibody binding to the leukocytes after stimulation. If this is the case the increased number of leukocytes positive for the platelet marker antibody (CAPP2A) binding may

represent random antibody binding to the leukocytes rather than platelets actually bound to leukocytes. Cellular visualization could potentially be used to confirm the presence of platelets on leukocytes. In future work the use of fluorescently conjugated anti-monocyte, anti-platelet, or anti-granulocyte antibodies would enhance the reproducibility of the assay, although such antibodies are not yet available for ovines. Also important to note is the importance of using ACK buffer to lyse the red blood cells. The use of water will also lyse ovine red blood cells, but in the process appears to lyse granulocytes and other leukocytes making it inappropriate for the study of leukocyte activation. At this point caution must be used in applying these LPA assays in vivo.

3.3 LYMPHOCYTE ACTIVATION ASSAY DEVELOPMENT

3.3.1 Methods

There were several commercially available antibodies that were specific for ovine lymphocyte activation antigens, which we sought to evaluate for their potential to recognize ovine lymphocyte activation after stimulation. These included an anti-ovine CD25 antibody, and antibodies that bind to the ovine DR and DQ subunits of MHC class II. Whole blood was collected from sheep via jugular venipuncture, discarding the first 3 mL and added to sodium citrate tubes. Blood was incubated with rat smooth muscle media that contained antibiotics: gentamicin, and streptomycin in 12 well plates. Heparin (11 U/mL, final concentration) was added along with the lymphocyte agonists: Concanavalin A (5 μg/mL, Con A), or Phorbol-myristate acetate (0.2 μM, PMA). In order to induce expression of activation epitopes the

blood/media mixture was incubated for 95 hrs in a 37°C incubator. After incubation blood/media mix (200 μL) was then incubated with Tyrode's buffer with citrate (115 μL), anti-ovine CD4 antibody (to label CD4 T-cells; 5 μL), and 5 μL of either the potential lymphocyte activation antibody (anti-ovine CD25 antibody, anti-ovine MHC–DR, or anti-ovine MHC-DQ) or the isotype control antibodies (IgG_1 or IgG_{2a}) into polystyrene tubes for 20 min. Samples were then lysed with 2 mL of ACK buffer, centrifuged and then washed with 1 mL citrated tyrode's buffer, and then fixed with 1% paraformaldehyde. CD4 positive T-cells were analyzed for the fluorescence of the respective activation antibody (anti-ovine CD25, anti-ovine MHC-DR, or anti-ovine MHC-DQ) on the flow cytometer. Positive events were determined by setting a mark that excluded 98% of the isotype control antibody fluorescence. IgG_1 was used as the isotype control antibody for anti-ovine CD25 and anti-ovine MHC-DQ. IgG_{2a} was used as the isotype for anti-ovine MHC-DR. Results from these studies are shown in **Figure 3-7**.

3.3.2 Results

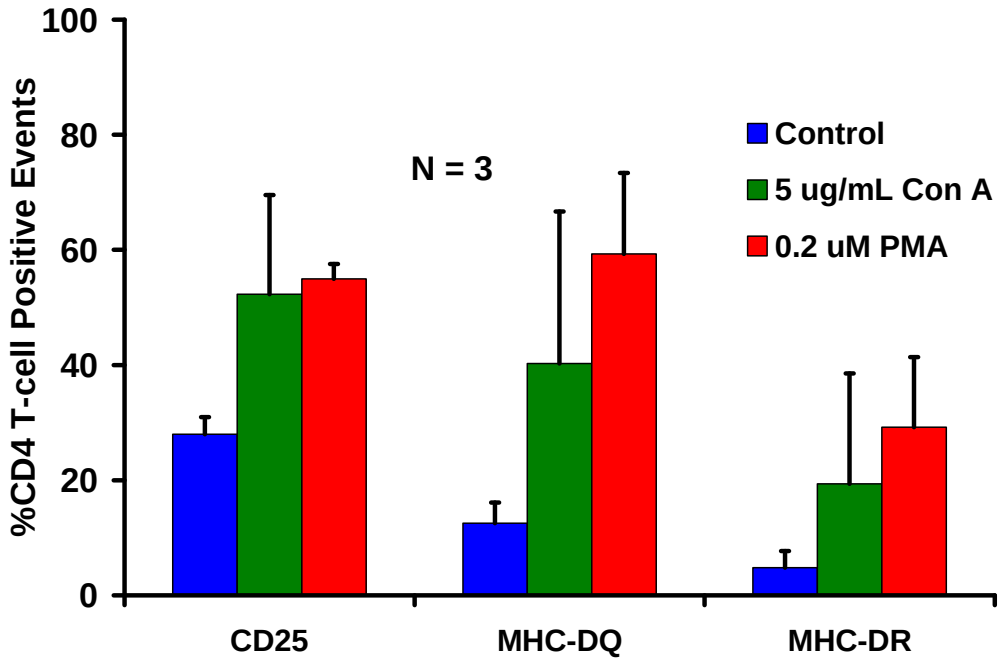

Figure 3-7 CD4 T-cell Activation following stimulation

3.3.3 Discussion

With each antibody, there is increased binding to CD4 T-cells following stimulation with the Con A or PMA agonists. In the case of anti-ovine CD25 and anti-ovine MHC-DQ, the control binding is somewhat high. This maybe in part due to a lymphocyte reaction with the polystyrene over the 95 hr time period that induced some expression of protein epitopes; however, CD25 is constitutively expressed on some CD4 T-cells that are commonly referred to as regulatory T-cells. The anti-ovine MHC-DR antibody also appears to be promising. However

the fluorescence associated with its isotype control (IgG_{2a}) also rose in a similar fashion, and raises the question if the anti-MHC-DR antibody is actually binding to a meaningful cell surface antigen as was the concern with leukocyte platelet aggregates assay. Also in question is the IgG_{2a} isotype control antibody, because the IgG_1 antibody used as the isotype for analysis of the anti-CD25 and MHC-DQ antibody did not increase to a high degree in the cultures with the Con A and PMA agonists. It may be possible that the IgG_{2a} antibody is recognizing an epitope on T-cells that might make it inappropriate for use as an isotype in this lymphocyte activation assay. Of note, increased binding with IgG_{2a} antibody has been mentioned by other flow cytometry users.

3.4 CONCLUSIONS

Antibodies that recognize ovine monocytes and granulocytes were tested and could distinguish these cells. Granulocyte and monocyte platelet aggregate assays were also developed but their utility is in doubt given the results from the negative control antibody following stimulation. CD4 T-cell activation assays that quantify CD25, MHC class II DQ, and MHC class II DR expression on cells were evaluated in vitro and were shown to bind to activated ovine lymphocytes and should be useful (in particular the CD25 and MHC DQ assays) in evaluating in vivo ovine lymphocyte activation following cardiovascular device implant.

4.0 BIOCOMPATIBILITY ASSESSMENT OF THE FIRST GENERATION PEDIAFLOW DEVICE

4.1 INTRODUCTION

As mentioned in **Section 1.4** the University of Pittsburgh consortium was awarded a contract from the NHLBI pediatric circulatory support program to build the PediaFlow™ pediatric VAD [5, 12, 18, 113]. The PediaFlow VAD is a mixed flow turbodynamic VAD that employs a magnetic suspension [5, 18, 114]. The ultimate goal of this device is to deliver a flow rate between 0.3 and 1.5 L/min to serve a patient population from newborns to approximately 2 year olds. The device aims for an implantation period of up to 6 months to provide cardiac support as a child awaits a transplant or myocardial recovery. The first generation design (PF1) weighs approximately 100 g and pumped a maximum of 660- 810 mL/min against physiologic pressure in three ovine animal studies. It measures 51 mm in length, has a 28 mm outer diameter and a pump priming volume of less than 2 mL. In concert with meeting the clinical design requirements stated above, the focus of PediaFlow VAD development is to achieve high levels of blood biocompatibility. To achieve this criterion, the PF1 flow path was developed using iterative computational fluid dynamics (CFD) to minimize areas of high shear and to possess smooth velocity vectors devoid of stagnation or recirculation zones [5, 18, 113, 115]. Heat generation caused by the pump was quantified and determined to be within acceptable limits (≤

2°C temperature rise) during normal operation [116]. Furthermore, magnetic bearings were chosen for the PediaFlow VAD to avoid the wear and heat generation associated with contact bearings [117-118]. Since contact bearings are also known to form a high shear region and may promote hemolysis as well as thrombus formation, the use of magnetic bearings in the PediaFlow VAD, it was hypothesized, would significantly improve its potential for excellent biocompatibility *in vivo* [117-119].

Damage to red blood cells (hemolysis) and platelet activation are important parameters that are often measured to assess the biocompatibility of artificial organs [44, 62-63, 100, 120]. The use of platelet biocompatibility assays has demonstrated utility in evaluating VAD design, as described above; providing guidance to potentially improve device hemocompatibility [62-63]. To assess the biocompatibility of the PediaFlow PF1 design in this report we employed assays for hemolysis (quantified by plasma free hemoglobin; plfHb), and plasma protein and fibrinogen concentrations (all courtesy of Dr. Marina Kameneva), in addition to flow cytometric assays to quantify ovine platelet activation (discussed in **Chapter 2**) during implantation in vivo [80, 120].

4.2 METHODS

In vitro biocompatibility flow loop test

Blood collection

Ovine whole blood (540 mL) was collected by jugular venipuncture using an 18 gauge 1.5-in. needle with syringe and stopcock into a blood bag containing 60 mL of anticoagulant

citrate dextrose (ACD) solution. Heparin (4 U/mL) was added to the blood reservoir post collection (hematocrit = 28%).

Flow loop setup

A fluid dynamic test loop with a blood bag reservoir and the PF1 pediatric VAD was prepared with luer ports for pressure measurement at VAD inlet and outlet as well as blood temperature measurement. A magnetic stir bar was introduced in the bag, which was placed on a magnetic stirrer to prevent blood stagnation. The circuit was cleaned and the surfaces passivated by introducing, in sequence, detergent (Simple Green, Sunshine Makers, Inc., Huntington Beach, CA), enzymatic detergent (Tergazyme, Alconox, Inc., White Plains, NY), water (twice), 1% bovine serum albumin solution in Tyrode's Buffer, and saline. Each of these fluids was pumped by the PF1 pediatric VAD through the test loop at minimum speed (3,000 rpm) for 15 min. Ovine blood (500 mL) was then added to the reservoir and circulated at 650 – 750 mL/min for 6 hours at 10,500 rpm. Resistance of the circuit (a tubing clamp downstream of the pump) was set to achieve an afterload pressure of 80 mmHg. The blood bag was placed on a magnetic stirrer and a magnetic stir bar was inserted in the blood bag for constant mixing of the blood reservoir. A bag with the same ovine blood (~100 mL) was slowly rocked next to the circulation loop to serve as a control. A blood sample was taken at the start of the experiment immediately after the pump reached maximum speed (hour 0). The pump operated for six hours and blood samples (3 mL) were collected hourly from the circuit and the control bag. Each time a blood sample was drawn from the flow loop the volume was replaced with blood from the control blood bag to maintain the flow loop blood volume at 500 mL.

Assessment of plasma free hemoglobin (plfHb)

Collected blood samples from both the control bag and the flow loop were centrifuged for 15 minutes at 2200 × g to obtain plasma. Plasma was transferred to microcentrifuge tubes and centrifuged at 20,800 × g for 20 minutes in a microcentrifuge (Eppendorf 5417R, Eppendorf North America, Westbury, NY). Plasma was then transferred to disposable semi-micro spectrophotometer cuvettes (Thermo Fisher Scientific Inc., Waltham, MA). PlfHb was measured for both samples at each time point using a spectrophotometer (Spectronic GENESYS 5, Thermo Fisher Scientific Inc.) at 540 nm wavelength [121]. The spectrophotometer was calibrated to zero using a blank solution according to established protocols. Hemoglobin calibration curve was obtained using standard dilutions of hemoglobin solution of known concentrations. "Blood damage" was then characterized by the normalized index of hemolysis (NIH) using the difference between the plfHb from the flow loop and from the control bag recorded at each time point [122].

In vivo testing

Surgical Procedure

Anesthesia was induced with ketamine and maintained with inhalation isoflurane. The characteristics of each implant can be found in **Table 4-1**. An arterial line for pressure measurement was placed in the left carotid artery along with a venous line for drug and fluid administration in the jugular vein. A left thoracotomy was performed through the fourth intercostal space. The inflow cannula was measured and cut to length and assembled to the PF1 inflow connector. A felt-coated sewing ring was fixed on the apex of the heart with pledgeted sutures and the cannula was inserted through this ring in the left ventricle via a stab wound. After

bolus administration of heparin (150 U/kg), the descending aorta was partially clamped using a vascular clamp (without cardiopulmonary bypass) and the outflow graft (**Table 4-1**) was anastomosed and fixed directly onto the pump outflow connector. The pump was started and set to run at maximum speed (**Table 4-1**). A 6PXL ultrasonic flow probe (Transonic Systems Inc, Ithaca, NY) was attached around the outflow graft. After closure of the chest, each animal was allowed to recover from anesthesia, and spontaneously ventilate. Marcaine (Hospira Inc, Lake Forest, IL USA) was used in the intercostal muscles and Banamine (Phoenix Pharmaceutical, Inc; St. Joseph, MO; 25 mg intravenously) was used for analgesia. Heparin was not administered for the first 48 hours following implant. Heparin was administered to maintain the ACT at approximately 180 seconds and was discontinued whenever the hematocrit dropped below 20%.

Blood collection

Pre-operative whole blood was collected from each ovine by jugular venipuncture using an 18 gauge 1.5-in needle with syringe and stopcock. The first 3 mL were added to sodium heparin tubes for plfHb and hemorheological parameter measurement. An additional 2.7 mL of blood for platelet activation assessment was drawn and added to tubes containing 0.3 mL of 0.106M trisodium citrate (Sarstedt, Newton, NC). In the 10-day implant an indwelling catheter was inserted for pre-operative blood collection. For this implant, samples were collected by withdrawing 20 mL of blood, then the sample volume, and then re-infusing the initial 20 mL of blood. Postoperative samples were collected daily through an indwelling arterial line that was placed during surgery for plfHb (reported in mg/dL) and hemorheology assessment. For platelet activation, samples were obtained on post-operative days 1, 2, and 3, and then twice weekly for the duration of each implant.

Assessment of blood and plasma parameters

Heparinized blood (3 mL) samples were used in hemorhelogical assays to measure blood parameters including plasma free hemoglobin, total blood hemoglobin, hematocrit, fibrinogen and total plasma protein concentrations. Hematocrit was determined in a microhematocrit centrifuge (IEC MB Centrifuge, International Equipment Company, Needham Hts, MA). Total blood hemoglobin concentration was measured in a hemoximeter (ABL 700 Series, Radiometer American Inc., West Lake, OH). The remaining sample was centrifuged at 9500 × g for 15 min at room temperature (ML Vanguard V6-500, Marketlab, Inc., Caledonia, MI). The supernatant was then added to a 1.5 mL microcentrifuge tube and centrifuged again (Laboratory Centrifuge IEC MiniMax, International Equipment Company, Needham Hts, MA) at 15,000 × g for 12 min. The supernatant was transferred to a 1.5 mL microcentrifuge tube and then centrifuged at 20,800 × g for 20 min (Eppendorf 5417R, Eppendorf North America). The resulting plasma was then transferred to disposable semi-micro cuvettes (Thermo Fisher Scientific Inc.) for measurement of plasma free hemoglobin as described above. Plasma total protein and fibrinogen concentrations were assessed using a benchtop refractometer (Kernco Instruments Co. Inc., El Paso, TX). Fibrinogen provided an indirect assessment of the inflammatory state of the animal and plasma protein was used as an indirect measure of volume status and is also generally related to liver function.

Table 4-1: PF1 implant summary

In vivo study #	Animal weight (kg)	Duration of support	Inflow cannula	Outflow cannula	Flow rate (L/min)	Pump Speed (krpm)	Mean Arterial Pressure (mm Hg)
1	54	6 d	shortened 20Fr DLP	6 mm Vascutek	0.81	10.7	76.5
2	30	17 d	shortened 20Fr DLP	PVC + 6 mm Vascutek	0.55	11.0	94.7
3	34	10 d	shortened 20Fr DLP	PVC + 6 mm Vascutek	0.66	10.0	111.8

DLP cannula (Medtronic, Minneapolis, MN)
Vascutek outflow graft (Terumo Cardiovascular Systems Corp., Ann Arbor, MI)
PVC (6 mm) bonded to Vascutek graft by silicon

Assessment of platelet activation

Blood (5 μL) was transferred from tubes into 12 × 75 mm polystyrene tubes with 5 μL of 25 μg/mL of either coli S69 (isotype control, Washington State University Monoclonal Antibody Center (Pullman, WA, USA)), MCA2419 (anti-human CD62P antibody, AbD Serotec, Raleigh, NC) or MCA2420 (anti-human CD62P antibody, AbD Serotec), 5 μL of 50 μg/mL goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), and 35 μL of Tyrode's buffer (Electron Microscopy Services, Hatfield, PA, USA) with 1% bovine serum albumin (BSA) and 0.106M sodium citrate and incubated for twenty minutes. The sample was washed with 1 mL of Tyrode's buffer with 1% BSA and 0.106 M sodium citrate (washing buffer) and mixed. Samples were then centrifuged for 10 min at 132 × g. The supernatant was removed and the pellet resuspended.

CAPP2A-biotin (5 μL of 7.5 μg/mL) and 5 μL of 73 μg/mL streptavidin-phycoerythrin (SA-PE; Invitrogen, Carlsbad, CA) were added to tubes and incubated for twenty minutes. Samples were then mixed with 1 mL of washing buffer and centrifuged at 132 x g. Following removal of the supernatant, samples were fixed with 1% paraformaldehyde.

Flow cytometric analysis was performed as described in **Section 2.1.2**. In the 10-day implant, platelet activation after stimulation was also evaluated. These samples were prepared as above using 25 μL of Tyrode's with 1% BSA, 5 μL of 20-mM glycine-proline-arginine-proline (GPRP for inhibition of fibrin polymerization, Anaspec, San Jose, CA, USA) in phosphate-buffered saline (PBS), and 5 μL of either 200 μM of adenosine diphosphate (ADP; Calbiochem, San Diego, CA, USA) or 100 μM platelet activating factor (PAF; Sigma-Aldrich; St. Louis, MO, USA) instead of the 35 μL of citrated Tyrode's buffer. In these stimulation studies the use of tyrode's without citrate along with GPRP is necessary as citrate will prevent the expression of additional p-selectin.

4.3 RESULTS

In vitro testing

Figure 4-1 illustrates the PediaFlow PF1 pediatric VAD with its outer housing (top panel), without its outer housing (middle panel), and a close-up view of the impeller (bottom panel). In **Figure 4-2** the steady-state in-vitro pressure-volumetric flow rate curve of PF1 is shown using a 35% glycerine/saline (vol) blood-analog for pump speeds of 4600-10000 rpm. During the 6 hr *in vitro* blood test, the pump successfully generated a mean flow of 700 mL/min

against an 80 mm Hg afterload at 10,600 rpm. The mean hourly NIH value was 0.0087 ± 0.0024 g/100L, a clinically acceptable result [122].

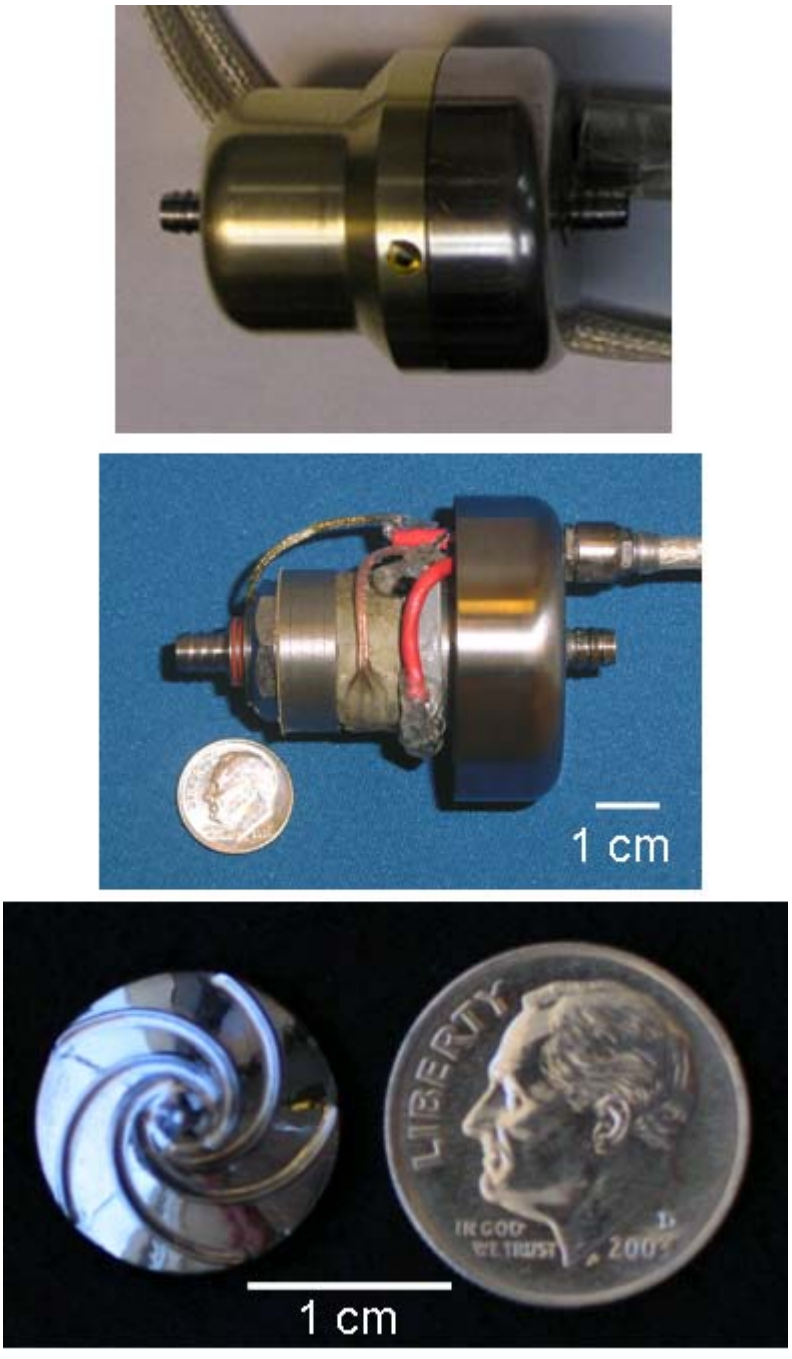

Figure 4-1: Images of the PediaFlow PF1 pediatric VAD

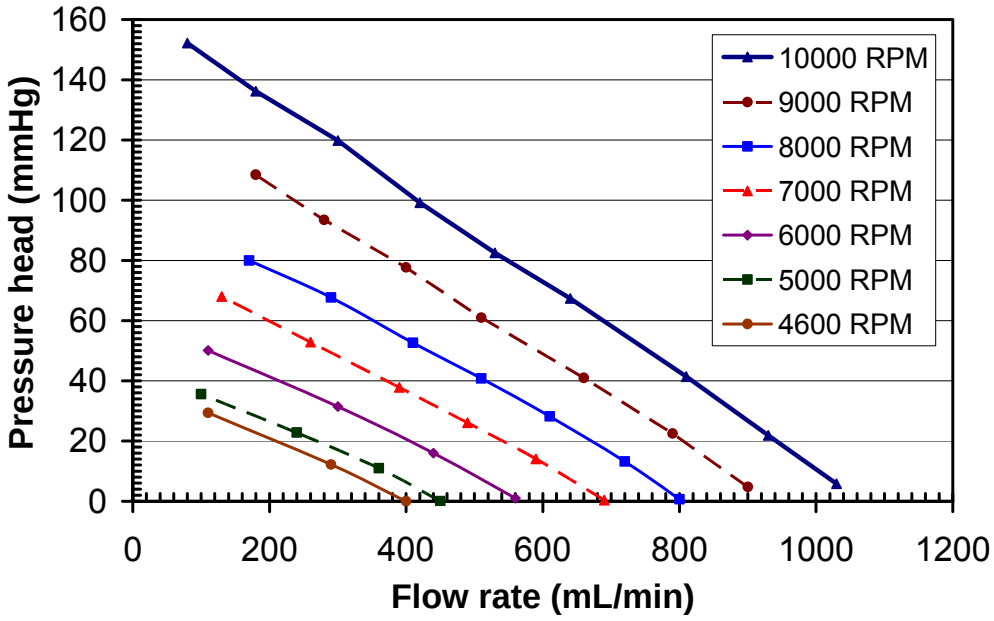

Figure 4-2: Pressure/Volume flow rate curve for PF1

The curve of PediaFlow PF1 was generated using a blood-analog for pump speeds of 4600-10000 rpm.

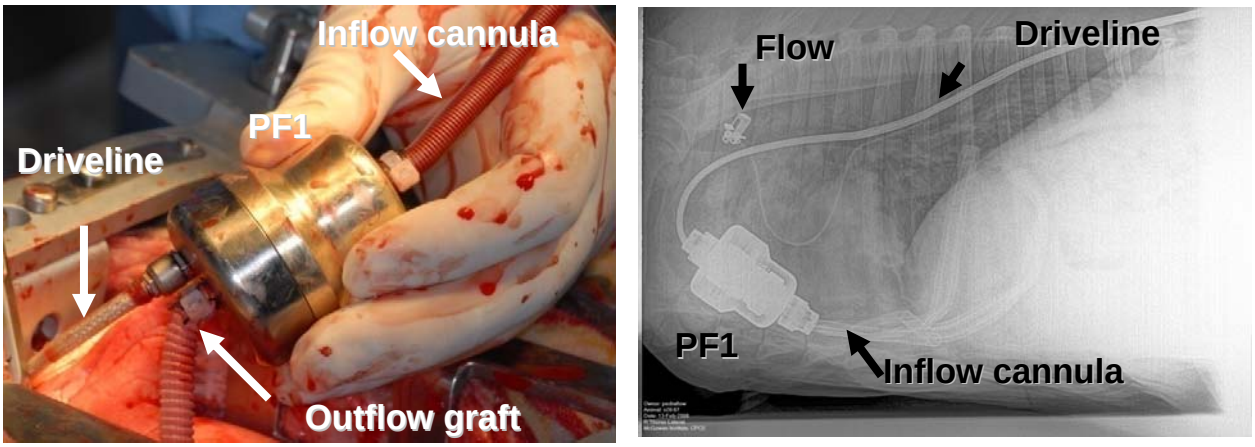

Figure 4-3 Image of the PF1 implant

A) Image of the PediaFlow PF1 during an implant procedure and B) Lateral chest radiograph following PF1 implant.

***In vivo* testing**

The implantation of the PF1 pediatric VAD is seen in **Figure 4-3A.** **Figure 4-3B** shows a lateral chest radiograph following PF1 implant. **Table 4-1** summarizes characteristics of each of the implants. At the end of study, there was a positive blood culture for bacteria in the first implant, although the white blood cell count was within normal limits. White blood cell counts were normal at the end of study for the other two implants and their blood cultures were negative for any microorganisms.

The flow rate and pump speed are plotted for each implant in **Figure 4-4.** In the first implant pump flow started off at 0.77 LPM and remained relatively constant concluding at 0.85 LPM. Pump speed was constant at a mean of 10.68 krpm. In the second implant pump flow was very low (0.3- 0.4 LPM) for the first five days, beginning to increase on day 6 and reaching its maximum (0.67 LPM) on day 9 and remained at approximately this value through the conclusion of the study. Pump speed in this implant starts at 10.6 krpm and begins to increase on day 9 and at the end of the study pump speed is approximately 11.5 krpm. Pump flow in the final study starts at a mean around 0.7 LPM. The flow signal is lost on days 4-7 due to a loss of acoustic coupling. The flow signal returns on day 8 and pump flow at the end of study is 0.6LPM, while pump speed is constant throughout the study at a mean of approximately 10 krpm.

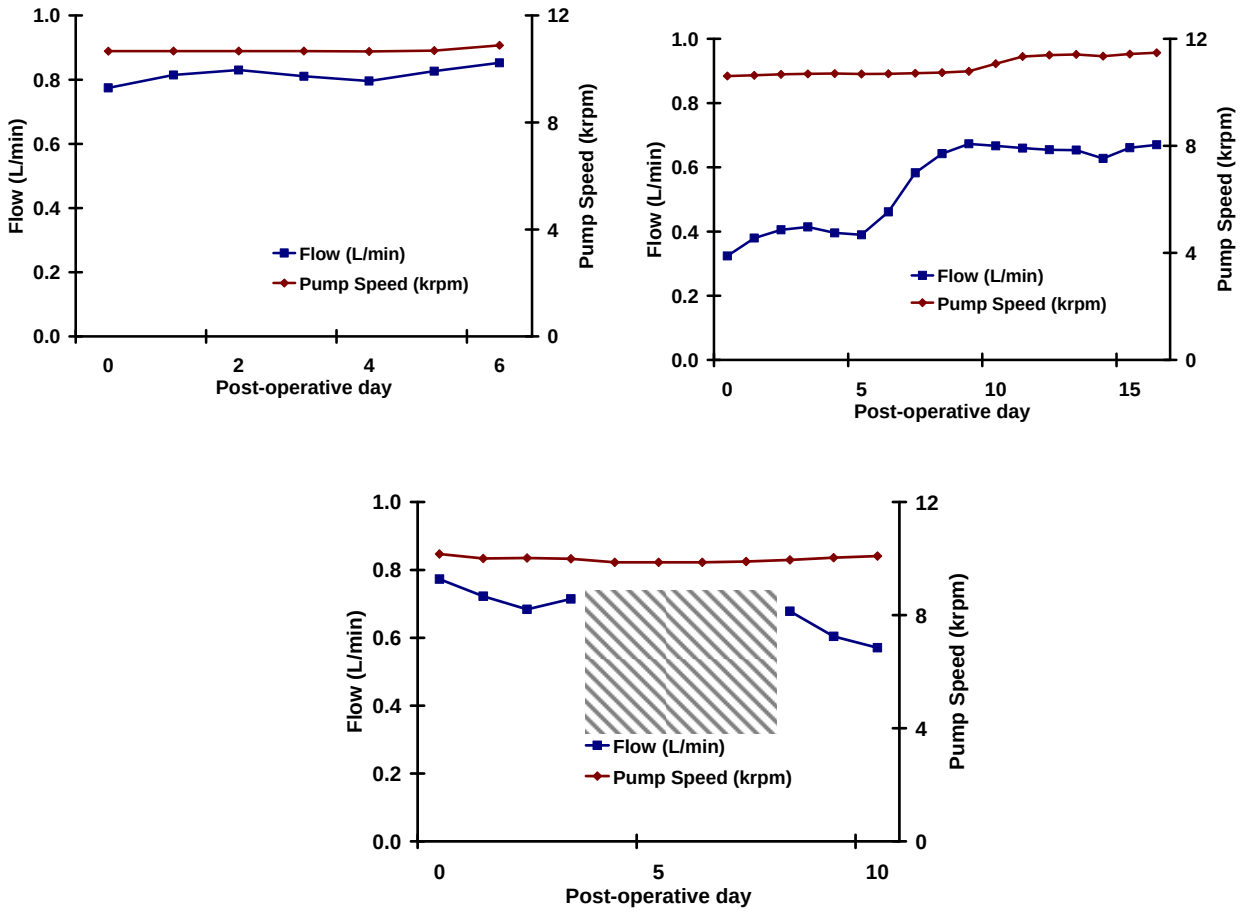

Figure 4-4 Flow rate and pump speed for the PF1 implants

A) first, B) second, and C) third PF1 implants (shaded area represents loss of acoustic signal).

In the first chronic study the outflow graft tore post-operatively because of a sharp cannula barb. This tear resulted in significant blood loss into the left chest with subsequent atelectasis which caused the animal to have labored breathing. The significant blood loss and respiratory compromise led to the implant being terminated on day 6. There were no renal infarcts or evidence of thromboembolism in other organs observed at necropsy. In **Figure 4-5** plfHb and hematocrit, total plasma protein and fibrinogen concentrations, and platelet activation results are presented for the first chronic implant. PlfHb was low for all 6 days of implantation with a mean value of < 5 mg/dL. The hematocrit decreased following the implant and began to

steadily decline on post-operative day 3 before a marked decrease on day 6 down to 10%. Total plasma protein concentration decreased post-operatively, but remained stable at approximately 5 g/dL for 5 days before decreasing to 4 g/dL on day 6. Fibrinogen concentration rose for the first 4 post-operative days (up to 700 mg/dL) and began to decrease on day 5. Platelet activation was very low at pre-operative values through day 2, but on day 3 rose markedly and remained elevated at the conclusion of the implant.

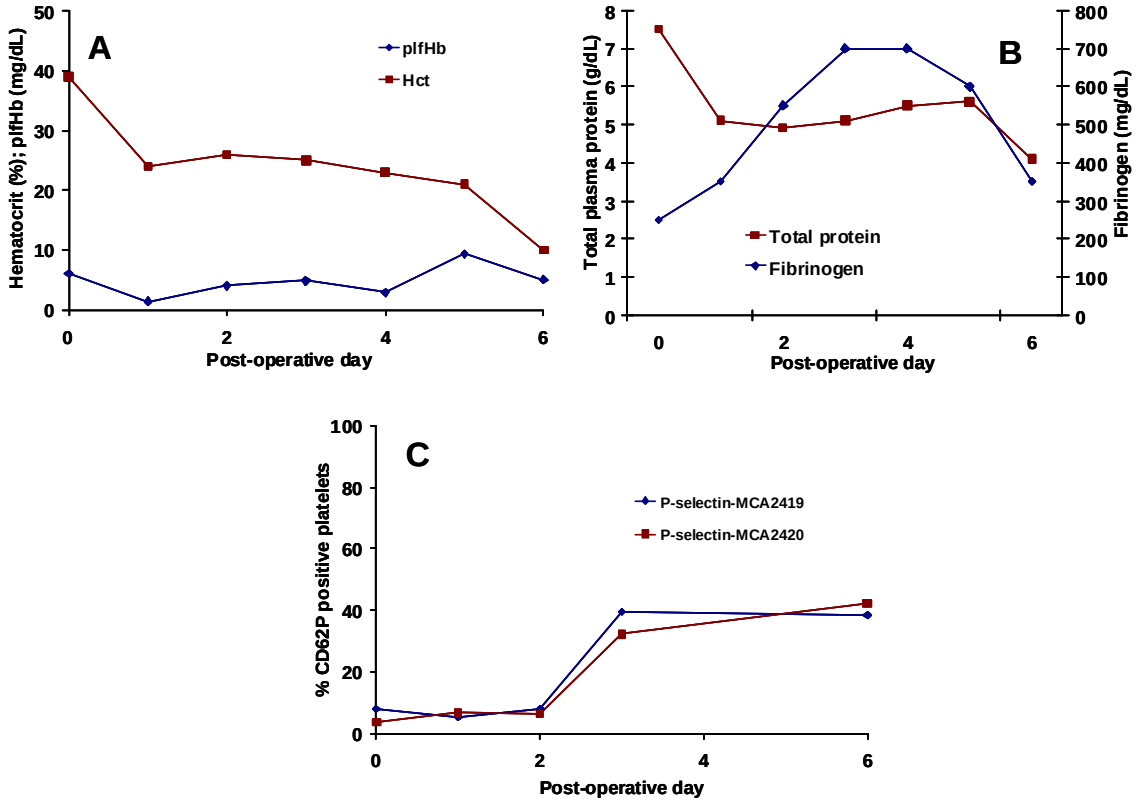

Figure 4-5 Blood parameter data for the first chronic PF1 implant

A) Hemolysis and hematocrit, B) total plasma protein and fibrinogen, and C) platelet activation (p-selectin expression on platelets) following the first PF1 chronic implant.

Technical challenges with the pump-cannula interface negatively impacted the second chronic implant resulting in the necessity of a very short inflow cannula with a sharp angle into

the pump, and a subsequently long, tortuous outflow graft anastomosed to the aorta. Initially very low pump outputs were observed for the target speed, which may have been due to kinking of the outflow graft which did eventually resolve. On day 15 there was a power outage in the animal facility, which, coupled with a failure in the PediaFlow uninterrupted power source, led to pump stoppage (15 min duration) and regurgitant flow through the pump. When power was restored, suspected thrombus embolization led to bowel and kidney infarction, which caused the gastrointestinal organs to fail and the animal to expire on day 17. Kidney, bowel, and gall bladder infarcts and an abnormal appearing liver were observed at necropsy. Left lung atelectasis and significant blood accumulation in the left chest were also observed. In **Figure 4-6** the plfHb and hematocrit, total plasma protein and fibrinogen concentrations, and platelet activation results for the second chronic implant are presented. The mean plfHb level for this implant was 13.3 ± 7.9 mg/dL. This parameter was substantially increased on days 4 through 9, but returned to baseline levels on day 10. The hematocrit steadily decreased following implantation and two blood transfusions were given to the animal (days 2 and 4). The hematocrit stabilized on day 4 at 20-22%. Total plasma protein concentration reduced post-operatively and began to rise on day 2 before stabilizing on day 4 at approximately 6 g/dL. The fibrinogen concentration significantly increased post-operatively (up to 700 mg/dL on days 2 and 3) and then slowly decreased returning to baseline on day 10. Platelet activation was elevated pre-operatively and was highly elevated after day 1.

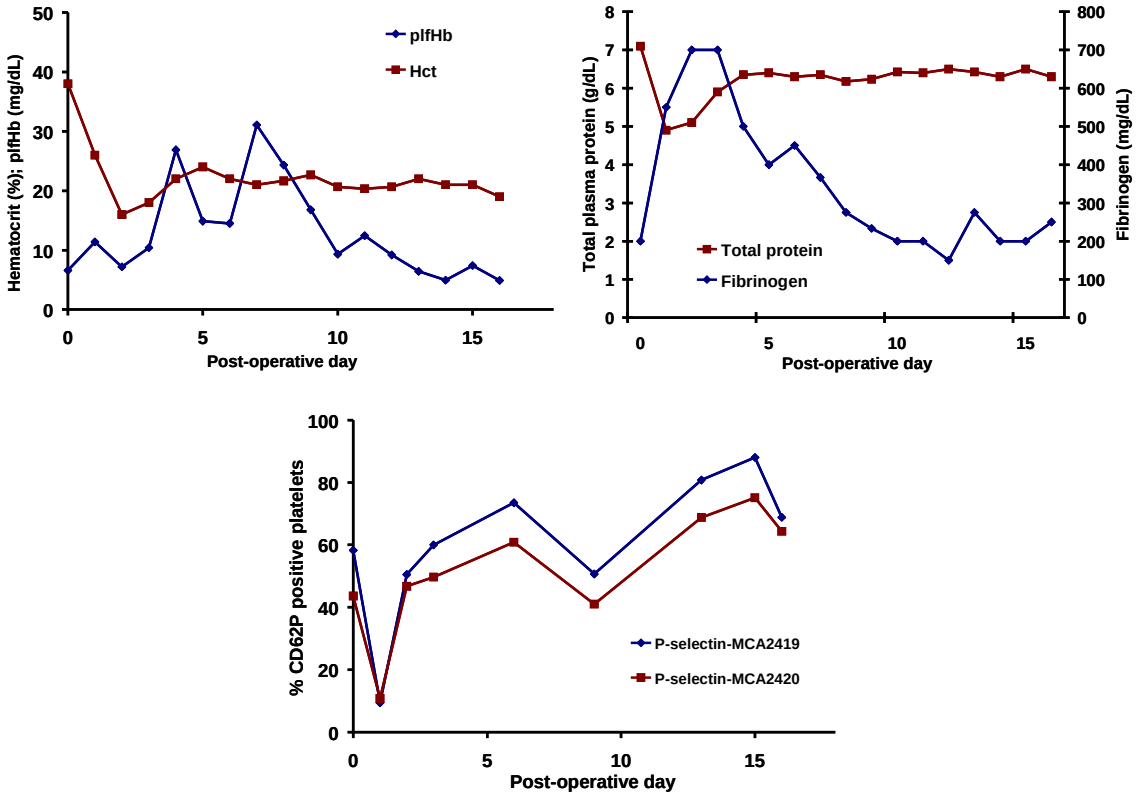

Figure 4-6 Blood parameter data for the second chronic PF1 implant

A) Hemolysis and hematocrit, B) total plasma protein and fibrinogen, and C) platelet activation (p-selectin expression on platelets) following the second PF1 chronic implant.

In our final PF1 implant technical challenges connecting the cannula with the pump resulted in a long partial aortic clamping time and was potentially the cause for the kidney infarct and the putative ischemic injury that prevented the animal from being able to stand post-operatively. On day 10 the percutaneous cable experienced an electrical short; the animal appeared to receive an electrical shock, then the pump failed and the implant was terminated. A healed renal infarct was observed in the right kidney at necropsy. At necropsy, several small tears were observed in the cable jacket insulation along with fluid entrapment, the likely cause of the electrical short. **Figure 4-7** illustrates the plfHb and hematocrit, total plasma protein and fibrinogen concentrations, and platelet activation results with and without exogenous stimulation

with ADP and PAF for the third chronic implant. The latter stimulation assays were newly developed and first assessed *in vivo* with our third animal**.** Mean plfHb for this implant was 8.8 ± 3.3 mg/dL. Hematocrit declined from its pre-operative value of 33% to 30% post-operatively and decreased slowly before stabilizing at approximately 25%. Total plasma protein concentration slightly decreased post-operatively but returned back to baseline on day 4. Fibrinogen concentration rose to extremely high levels of 1100 mg/dL on day 5 remaining at a very high level ~ 800-1000 mg/dl at the conclusion of the implant. Platelet activation rose slightly on days 1 & 2 and returned to baseline on day 4. Platelet activation began to rise on day 10 and sharply rose in the last data point on day 10. The mostly low platelet activation values in this implant were accompanied with an ability of the platelets to respond to application of agonists ADP and PAF for all but the final data point.

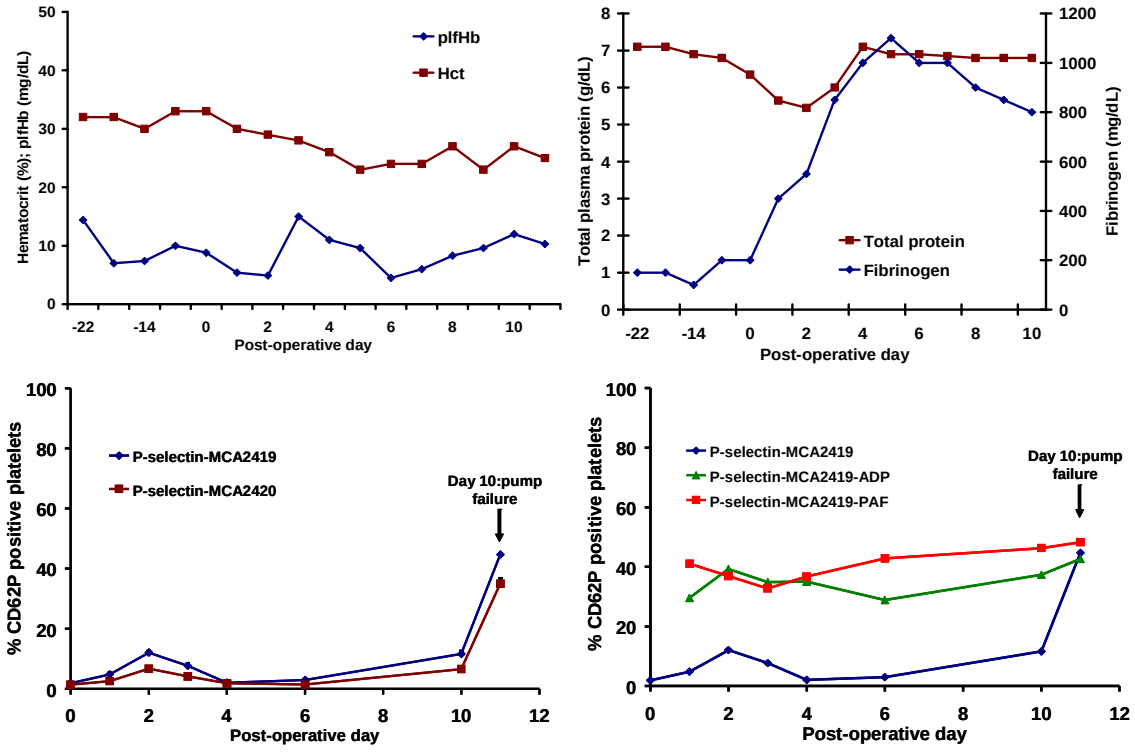

Figure 4-7 Blood parameter data for the third chronic PF1 implant

A) Hemolysis and hematocrit, B) total plasma protein and fibrinogen, C) platelet activation and D) platelet activation on platelets following stimulation of blood with 20 μM ADP and 10 μM PAF in the third PF1 chronic implant.

4.4 DISCUSSION

In all three ovine studies hematocrit decreased post-operatively and in the first two studies dropped precipitously. The precipitous drop in hematocrit in the first two studies may be explained by the observation of a large amount of blood in the left chest at necropsy. In the first study the drop in hematocrit was attributed to an outflow graft tear. In the second study however, no obvious tear sites in the graft or inflow cannula were observed, although there may have been bleeding at the pump/cannula connection site. Total plasma protein concentration also decreased post-operatively in each implant mostly due to blood dilution by the fluids given during surgery and post-operatively. The total protein rebounded during the post-operative course in the second and third studies. In the first implant however, total plasma protein concentration continued to decrease as hematocrit decreased down to 10%. Fibrinogen concentration rose in all three studies reflecting the normal post-surgical inflammatory reaction process seen in this animal model [23, 123]. In the first two studies fibrinogen began to decrease, returning to baseline levels. However, in the third implant it remained significantly above baseline at the time of implant termination. In this implant, the elevated fibrinogen might have been related to renal injury observed in this study as the creatinine in this animal was abnormally low, and the urea nitrogen value was near the lower limit of the normal range. Hepatic injury did not appear to be a concern in this implant because the AST (SGOT) value was normal.

Typically, a plfHb level > 10 mg/dL would be considered elevated; however, ovine cells are more fragile than human and bovine cells indicating that a slightly higher than 10 mg/dL plfHb level could still be considered satisfactory from the point of view of low potential mechanical damage to red blood cells produced by the pump [124]. Hemolysis levels were low in the first and third PediaFlow PF1 pediatric VAD studies. In the second implant the mean plfHb level was slightly above 10 mg/dL due to several spikes in plfHb value. The major contributor to the hemolysis was the suspected outflow graft kink; as the flow rate increased (presumably due to the kink resolving), the plfHb decreased to very low levels. The mean plfHb beginning on day 10 (pump reached its maximum flow on day 9) to the end of the implant was 7.8 ± 2.7 mg/dL. The need for several blood transfusions early in the post-operative period of this implant might have also impacted the elevated hemolysis levels observed early in the post-operative course. However, the baseline plfHb of the transfused blood was not measured before it was given to the sheep.

In the bovine model of preclinical testing, the utility of flow cytometric platelet activation assays has demonstrated value in differentiating between animals that had uneventful post-operative VAD courses and those that had evidence of substantial thromboembolism or pump thrombotic deposition [63-64]. Given our lack of experience in applying the developed ovine platelet activation assays from **Chapter 2** *in vivo*, we sought to understand how these assays responded during device implant and evaluate their utility for *in vivo* biocompatibility assessment. In the first implant, platelet activation rose in concert with an outflow graft tear that appeared to occur on day three and resulted in significant blood loss. During the second implant,

a kinked outflow graft significantly impeded flow and likely altered the flow path such that it became a nidus for platelet activation. The pronounced evidence of thromboembolic infarction observed at necropsy was reflected in the markedly increased sustained levels of platelet activation in this implant. Overall, the platelet activation data in the third chronic PediaFlow PF1 pediatric VAD implant was promising and was in contrast to the degree of platelet activation observed in the second chronic implant. The pump stopped on day 10 in the third implant as well, likely causing retrograde flow through the pump and presumably resulting in elevated platelet activation in the final blood collection data point. In all three studies platelet activation rose or had sustained elevation in response to pump complications (graft tear, kinked outflow graft and pump stoppage, pump electrical short and pump stoppage). The sensitivity of the platelet activation assays to pump complications in this limited data set suggests the utility of these assays as a meaningful temporal test of biocompatibility *in vivo*, in line with the bovine experience.

Platelet activation can be caused due to changes in the animal physiology or can be precipitated by pump complications. One way to attribute platelet activation to the pump is to perform sham surgeries. A sham surgery involves undergoing the equivalent surgery required for a VAD implant on a healthy animal without actual placement of the device. Characterizing platelet activation in sham studies then enables the determination of platelet activation attributable to this surgery. With a group of such sham surgical animals studied, one could characterize temporal platelet activation expected from the implant surgery and attribute excess or extended platelet activation as being related to pump placement and subsequent device operation. While earlier reports have performed such sham procedures with calf VAD implants,

we did not perform sham surgeries in this chapter [63]. It is also possible that for any animal a bleeding or thrombotic complication unrelated to the pump could arise in the implant period and that the platelet activation assays could detect this phenomenon. For these implants, given the noted pump-related complications, it seems likely that the elevations in platelet activation seen were related to those complications, but other sources cannot be entirely dismissed.

In the development of artificial organs, hemolysis is nearly universally assessed during preclinical testing, whereas platelet activation is not. These two parameters, both relevant to potential clinical-use blood trauma generated by a device, are not necessarily changing the same way over the course of device implantation. In the first implant of this study, hemolysis remained low throughout the study while platelet activation was elevated after day 2. In the second implant, hemolysis and platelet activation were both initially high, on day 10 however hemolysis returned to baseline levels, while platelet activation remained highly elevated. In the final implant hemolysis and platelet activation were both low until the final data point where platelet activation rose sharply following pump stoppage and the hemolytic markers remained relatively constant. These results illustrate the lack of agreement between the erythrocyte-related and platelet data. Considering the platelet activation data in concert with the hemolysis and blood protein data provides greater insight into the temporal course of VAD biocompatibility.

In the third implant the use of in vitro stimulation with agonists was introduced to evaluate circulating platelet responsiveness. A low platelet activation result following stimulation would suggest dysfunctional platelets which can not express its markers for platelet activation perhaps due to activation marker shedding. An inability for already activated platelets

to further respond to agonists would suggest a setting where circulating platelets are already activated to a point beyond which there is a limited ability to respond further. For the third implant the mostly low circulating platelet activation values were accompanied with an ability of these platelets to respond to agonists ADP and PAF. The response to these platelet agonists suggested that the low platelet activation levels observed in this implant reflected minimal impact of the PediaFlow PF1 pediatric VAD on platelets, suggesting the potential for good platelet biocompatibility. In contrast, the platelet activation value at the last data point in this implant was elevated and platelets from this data point were not able to respond further to stimulation.

In the second PF1 implant, pre-operative platelet activation was very high. To obtain pre-operative samples in our first two implants required animal restraint along with jugular venipuncture which induces fear in the animal. Turner and Hodgetts observed stress and anesthesia result in changes in ovine jugular hematocrit as a result of sequestering of red blood cells in the spleen [125]. The lack of tranquility in the sheep's environment was also mentioned as a potential stress [125]. These stresses then may have been responsible for the very high pre-operative platelet activation in our second PF1 implant. In the first PF1 implant the animal was housed in the animal facility for a much longer time than the animal of the second implant and had grown accustomed to its surroundings, perhaps contributing to the low pre-operative platelet activation values. To alleviate artifact in the collection of pre-operative platelet activation data, we have since adopted the practice of placing an indwelling catheter in the jugular vein pre-operatively in the implant animal. The use of such a catheter reduces the potential for stress-induced artifact in pre-operative sample collection by eliminating the need for animal

restrainment and needle stick associated with a jugular venipuncture blood draw. It is worth noting that post-operative samples are also collected through a vascular access point. The placement of the indwelling catheter pre-operatively was a potential cause for the low pre-operative platelet activation values observed in the third PF1 implant.

Pump flow was measured by an ultrasonic flow probe. The ultrasonic flow probe relies on a continuous and complete coupling between the probe and the outflow graft. The probe is initially coupled to the graft in the perioperative period with ultrasound jelly and serous fluids from the inflammatory response to surgery. The coupling is broken as these materials resorb, and there is a period of signal loss until tissue growth and encapsulation reforms the coupling, which in our experience typically occurs within 7-14 days post-operatively. This phenomenon should have been observed in the first two implants; however both of these implants unfortunately had significant blood loss into the chest and this fluid likely maintained continuous coupling.

The maximum flow rate for PF1 (0.81 LPM) was attained during our first implant. The design goal for the PediaFlow ultimately is to attain a maximum flow of 1.5 LPM. The PF1 was specifically designed to run at subcritical speeds, which limited our maximum speed and hence our maximum attainable flow. In our next PediaFlow design iteration we will focus on designing the motor (while maintaining the current fluid path) for supercritical performance which may enable us to attain the target flows. Once we have re-optimized the motor we can continue with additional implants to assess biocompatibility and hemodynamic performance. Although the fluid path will remain the same, the biocompatibility results may change from what we report in

this study. Our hope would be for improvement in platelet and red blood cell biocompatibility in future studies, however, our hemolysis and platelet activation assays have demonstrated their ability to be sensitive markers of biocompatibility whether the results are positive or not.

The chronic evaluation of the PF1 provided valuable guidance towards improving the PediaFlow pediatric VAD in future design iterations. The *in vivo* experience validates earlier CFD work and feasibility of the design of a magnetically levitated turbodynamic pump to generate flow rates in the range necessary to provide support to the youngest cardiac patients. The first generation pump design was able to generate promising low hemolysis data overall and at times, promising platelet biocompatibility. Attaining this level of biocompatibility at low flow rates is encouraging. Future design iterations of the PediaFlow however, must focus on achieving higher flow rates (1.5 L/min) in order to meet the design goal of providing cardiac support to children up to 2 years of age. Cannula/pump connection issues negatively impacted each implant, in particular the first two, and along with increasing the maximum flow rate these are the most significant issues to be addressed in the next design iteration. Pump miniaturization to ensure implantability in newborns and strengthening the cable jacket to prevent fluid penetration also needs to be addressed.

4.5 CONCLUSIONS

The PediaFlow™ PF1 pediatric VAD pumped 660-810 mL/min during three chronic ovine implants. While this 1st generation pediatric VAD design had several limitations with respect to a limited flow range, cannula connections, and compromised driveline, the use of

magnetic levitation and actuation to generate flow rates necessary to meet newborn patient cardiac demands was validated. Hemolysis levels overall were low during the implants. And circulating platelet activation assays temporally reflected pump/cannula implant problems, pump stoppage and animal post-operative complications, indicating the utility of these assays as sensitive markers of biocompatibility in the ovine model.

5.0 PLATELET ACTIVATION FOLLOWING IMPLANT OF THE SECOND GENERATION PEDIAFLOW DEVICE

5.1 INTRODUCTION

The second generation prototype of the PediaFlow VAD (PF2; shown in **Figure 5-1**) was able to achieve higher flow rates than our first generation PediaFlow VAD through supercritical operation by utilizing a more efficient 4-pole motor. Although the fluid path remained the same, the outer housing has a significantly reduced device volume of 35.3 mL (40% decrease) and improved cannula connections when compared to the first generation PediaFlow design. Our objective in this chapter was to characterize platelet activation during the implant and operation of the second generation design of the PediaFlow VAD and also perform a series of surgical sham studies to examine purely surgical effects from the implantation procedure. Both circulating platelet activation status and the ability of circulating platelets to respond to agonists delivered in vitro were quantified. In addition a newly available antibody marker to quantify ovine platelet activation was characterized as was the effect of obtaining pre-operative blood from venipuncture versus an indwelling venous catheter.

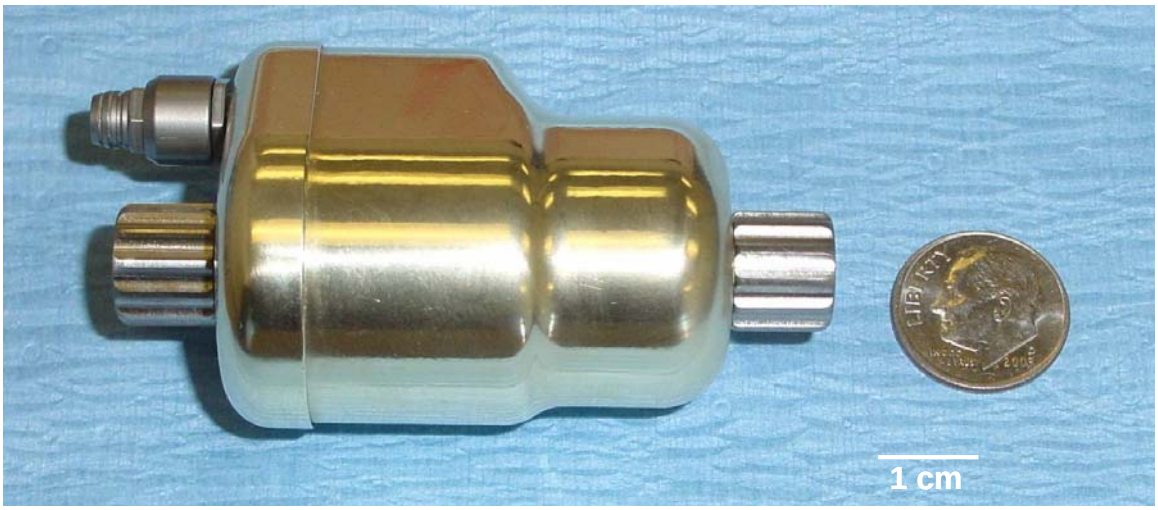

Figure 5-1 The PediaFlow PF2 pediatric VAD

5.2 METHODS

Blood Collection

An indwelling catheter was inserted in the jugular vein of Dorset-cross, Cheviot, and Suffolk sheep for pre-operative blood collection for each of the PF2 implants and surgical sham studies. At least three pre-operative samples were collected from the indwelling catheter and averaged to obtain the pre-operative data point (post-operative day zero).

Pre-operative blood samples were also used to assess the ability of MCA2418 (an anti-human CD62P antibody-clone Psel.KO.2.5; AbD Serotec; Raleigh, NC, USA) to preferentially bind to activated ovine platelets in nine ovines. To assess the effect of the jugular vein catheter versus jugular venipuncture on pre-operative platelet activation, blood was also drawn via jugular venipuncture as described in **Section 2.1.2.**

In vitro biocompatibility flow loop setup

A mock circulatory test loop with a blood bag reservoir and including the PF2 pediatric VAD was prepared as described in **Section 4.2**. Ovine blood (240 mL) was added to the reservoir and circulated for 2 hr each at 0.5, 1.0, and 1.5 L/min. Blood was collected hourly for assessment of platelet activation. A similar test loop was constructed with another prototype pediatric VAD device which utilized an axial thrust bearing (Toddler VAD, **Figure 8-6)** for comparison and evaluated for one hr at 1.8 L/min.

Assessment of platelet activation

Platelet activation was quantified during the 6 hr in vitro study before and after exogenous stimulation with platelet activating factor (PAF; Calbiochem, San Diego, CA, USA). Platelet activation was assessed pre and post surgery with samples from each PF2 implant and sham surgery animal. In addition, platelet activation was assessed following stimulation using adenosine diphosphate (ADP; Calbiochem, San Diego, CA, USA) or PAF in samples from three of the sham animals and from all of the PF2 implants.

Blood (5 μL) was transferred from tubes into 12 × 75 mm polystyrene tubes with 5 μL of CAPP2A (7.5 μg/mL; Veterinary Medical Research and Development; VMRD, Pullman, WA, USA), 5 μL of goat anti-mouse IgG-phycoerythrin (60 μg/mL; Invitrogen, Carlsbad, CA, USA), and 35 μL of Tyrode's buffer (Electron Microscopy Services, Hatfield, PA, USA) with 1% bovine serum albumin (BSA) and 0.106M sodium citrate and incubated for 20 min. Each unstimulated sample was performed in duplicate.

ADP and PAF stimulated samples were prepared as above using 25 µL of Tyrode's buffer with BSA, and 5 µL of either 200 µM ADP or 100 µM PAF, and 5 µL of 20-mM GPRP (Anaspec). Samples were then washed with 1 mL of Tyrode's buffer with 1% BSA and 0.106 M sodium citrate (washing buffer) and mixed. Samples were then centrifuged for 10 min at 132 × g. The supernatant was removed and the pellet resuspended. IgG1-Alexa Fluor 488 (isotype control; 5 µL of 25 µg/mL; MCA928A488, AbD Serotec), MCA2418-Alexa Fluor 488 (5 µL of 25 µg/mL; MCA2418-A488), or MCA2419-Alexa Fluor 488 (5 µL of 25 µg/mL; anti-human CD62P-clone Psel.KO.2.7, AbD Serotec) were then added to tubes and incubated for twenty min. Samples were then mixed with 1 mL of washing buffer and centrifuged at 132 x g. Following removal of the supernatant, samples were fixed with 1% paraformaldehyde. Flow cytometric analysis was performed as described in **Section 2.1.2**. MCA2419 was utilized as the platelet activation marker to quantify the effect of the jugular vein catheter on pre-operative platelet activation.

In vitro characterization of MCA2418 binding to activated ovine platelets

MCA928-A488 was used as the isotype control antibody for the evaluation of MCA2418-A488. Unstimulated samples were prepared as above replacing the 35 µL of citrated Tyrode's buffer with 30 µL of Tyrode's buffer with (BSA) and 5 µL of GPRP. ADP and PAF stimulated samples were prepared as above. Fluorescence associated with MCA2418-A488 for control and activated samples was compared to evaluate whether MCA2418 preferentially bound to activated ovine platelets. Flow cytometric analysis was performed as described in **Section 2.1.2**.

Implant procedure

Three sheep were implanted with two PF2 prototypes (PF2.1, PF2.2- **Table 5-1**). A problematic sensor wire (discussed below) was replaced between the first and second PF2.1 implants. Implant procedure and post operative care was performed as in **Section 4.2** with the following alterations. The inflow cannula was an 18F cardiopulmonary bypass cannula (DLP; Medtronic, Minneapolis, MN, USA) trimmed to 7 cm and communicated with the left ventricle through a stab incision to the heart and secured with a felt-coated sewing ring fixed on the apex of the heart with pledgeted sutures. The outflow cannula (20F EOPA cannula trimmed to 9 cm, Medtronic) was inserted by an introducer through a snare incision into the descending aorta and the cannula was then fixed directly onto the pump outflow connector. The pump was started and operated at the desired RPM (**Table 5-1**). In addition to heparin administered to maintain the activated clotting time between 180-200 secs. Warfarin (intravenous coumadin; Bristol-Myers Squibb; Princeton, NJ, USA) was also used to maintain the INR in the range of 2.0-3.0.

Table 5-1: PF2 implant summary

Study #	Pump Prototype	Length of Study (Days)	Average Pump Speed (krpm)	Estimated Average Pump Flow (L/min)
1	2.1	17	13.5	1.2
2	2.1	30	15.5	1.5
3	2.2	70	8.5	0.5

Sham surgeries were performed on five animals as described for the implant with the following alterations: No inflow cannula was inserted into the sewing ring. After the stab incision into the apex of the left ventricle the sewing ring site was stapled shut. The descending

aorta was partially clamped with a vascular clamp and an outflow graft was anastomosed onto the aorta and tied off at the anastomotic site. Coumadin was not used in the sham studies.

Statistical Analyses

All data are presented as mean with standard deviation. Statistical analyses were performed using SPSS 12.0.1 (SPSS, Chicago, IL, USA). One-way analysis of variance with repeated measures (Bonferroni post hoc test) was used to compare the means of MCA2418 binding to unstimulated and stimulated ovine platelets**.** A paired samples t-test was used to compare means of pre-operative platelet activation from jugular venipuncture and jugular venous catheter samples. Significance was considered to exist for $p<0.05$.

5.3 RESULTS

Effect of venous line placement and in vitro assessment of platelet activation

In **Figure 5-2** the MCA2418 antibody bound at significantly higher levels to ADP and PAF-stimulated ovine platelets when compared to unstimulated ovine platelets. Pre-operative platelet activation after jugular venipuncture and following placement of a jugular venous catheter is shown in **Figure 5-3**. The level of platelet activation measured in samples obtained from the venous catheter was significantly reduced from those drawn through jugular vein venipuncture. There was also less variance observed in these activation levels when the catheter was utilized.

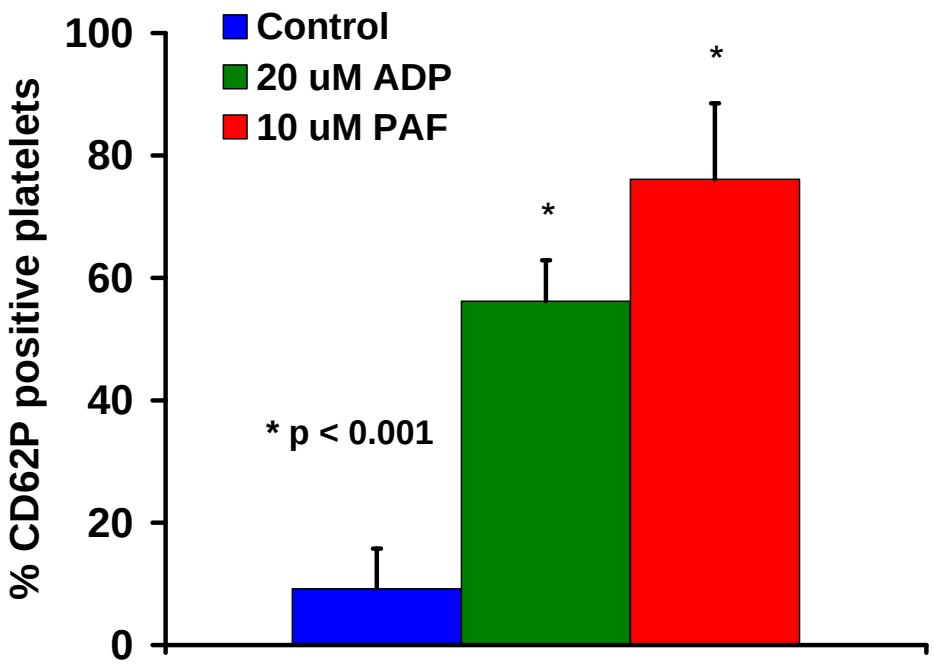

Figure 5-2 MCA2418 binding to ovine platelets

CD62P positive platelets quantified by MCA2418 antibody binding to control and 20 μM ADP and 10 μM PAF-stimulated platelets (N = 9). MCA2418 binding to ADP and PAF stimulated platelets was significantly higher ($p < 0.001$) then its binding to unstimulated platelets.

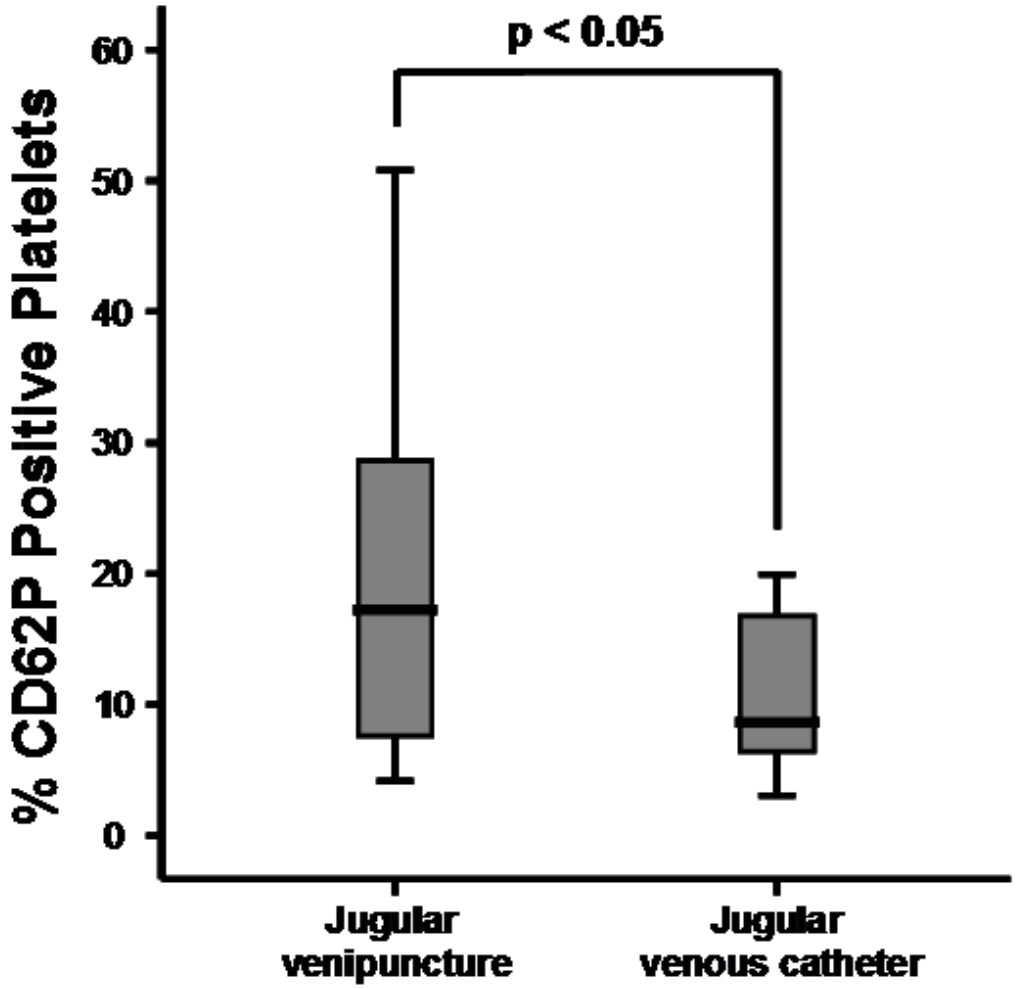

Figure 5-3 Comparison of method of pre-operative blood collection

Boxplot comparison of pre-operative platelet activation from blood taken by jugular venipuncture and after insertion of a jugular venous catheter (N = 12 each). Platelet activation following placement of the jugular vein catheter was significantly lower ($p < 0.05$) then platelet activation from jugular venipuncture samples.

Platelet activation during a 6 hr in vitro study with the PF2.2 VAD is illustrated in **Figure 5-4**. CD62P-positive platelets remained low (< 11%) during this entire study; and the percentage of CD62P-positive platelets increased markedly following stimulation with 10 μM PAF throughout the study. In contrast platelet activation following one hr of contact with the other

pediatric pump employing the axial thrust bearing rose to 18% in one hr and platelet activation following PAF stimulation rose only to 22%.

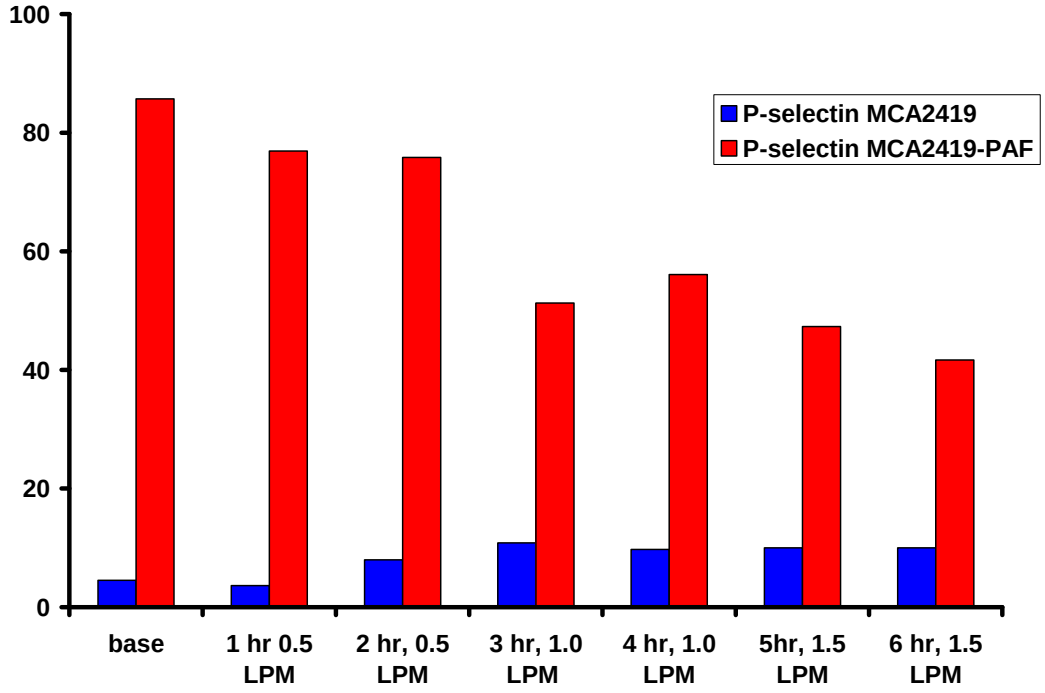

Figure 5-4 PF2 in vitro platelet activation test

A) Platelet activation during a six hr in vitro circulatory loop with the PF2.2 pump before and following stimulation with 10 μM PAF.

Sham surgery studies

In **Figure 5-5A** platelet activation quantified by MCA2419 rose following sham surgery; reaching a peak on day 7 and then steadily declining, returning to baseline levels within the first 17 days. In **Figure 5-5B** platelet activation quantified by MCA2418 with and without agonist stimulation of the drawn sample is presented. Platelet activation increased following stimulation with ADP and PAF but the difference between stimulated and unstimulated activation levels was diminished early in the post-operative period compared to the pre-operative difference. The difference in platelet activation before and after stimulation increased as surgery-associated

platelet activation dissipated. No kidney infarcts were observed at necropsy in any of these sham surgery animals.

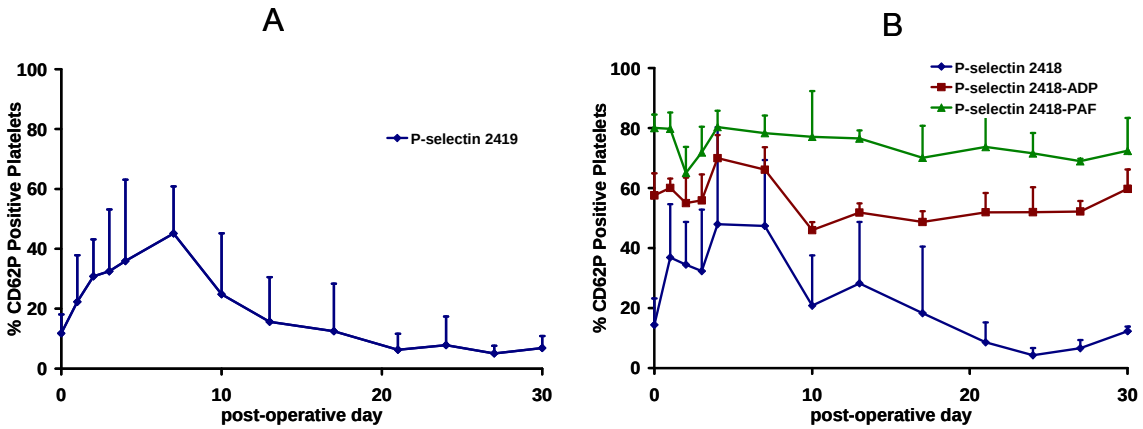

Figure 5-5 Platelet Activation following sham studies

A) Platelet activation in five sham surgical studies (n = 5) and, B) platelet activation in sham surgical studies following stimulation of blood with 20 μM ADP and 10 μM PAF (N = 3).

Ovine implants of the PF2 VAD

Figure 5-6 shows platelet activation results and necropsy images from the 17-day PF2.1 VAD implantation. Platelet activation rose following surgery reaching a maximum on day 3 and returned to baseline levels on day 6. Platelet activation remained low before sharply rising on day 16 subsequent to numerous pump stoppages (rotor de-levitation) that occurred prior to termination of the study. These events caused rotor touchdown and were found to be the result of a manufacturing defect in a sensor wire in the percutaneous cable. In **Figure 5-6B** platelet activation following agonist stimulation was elevated above platelet activation found in unstimulated blood over the course of the implant. As with the sham surgery animals, this difference in activation increased as unstimulated platelet activation returned to baseline levels.

However, in the final samples taken after the pump stoppages the difference between unstimulated and stimulated samples again decreased. As seen in **Figure 5-6C**, no obvious kidney infarcts were observed at necropsy and the outlet region of the impeller had a large number of scratches covering the surface that were attributable to the numerous pump stoppages.

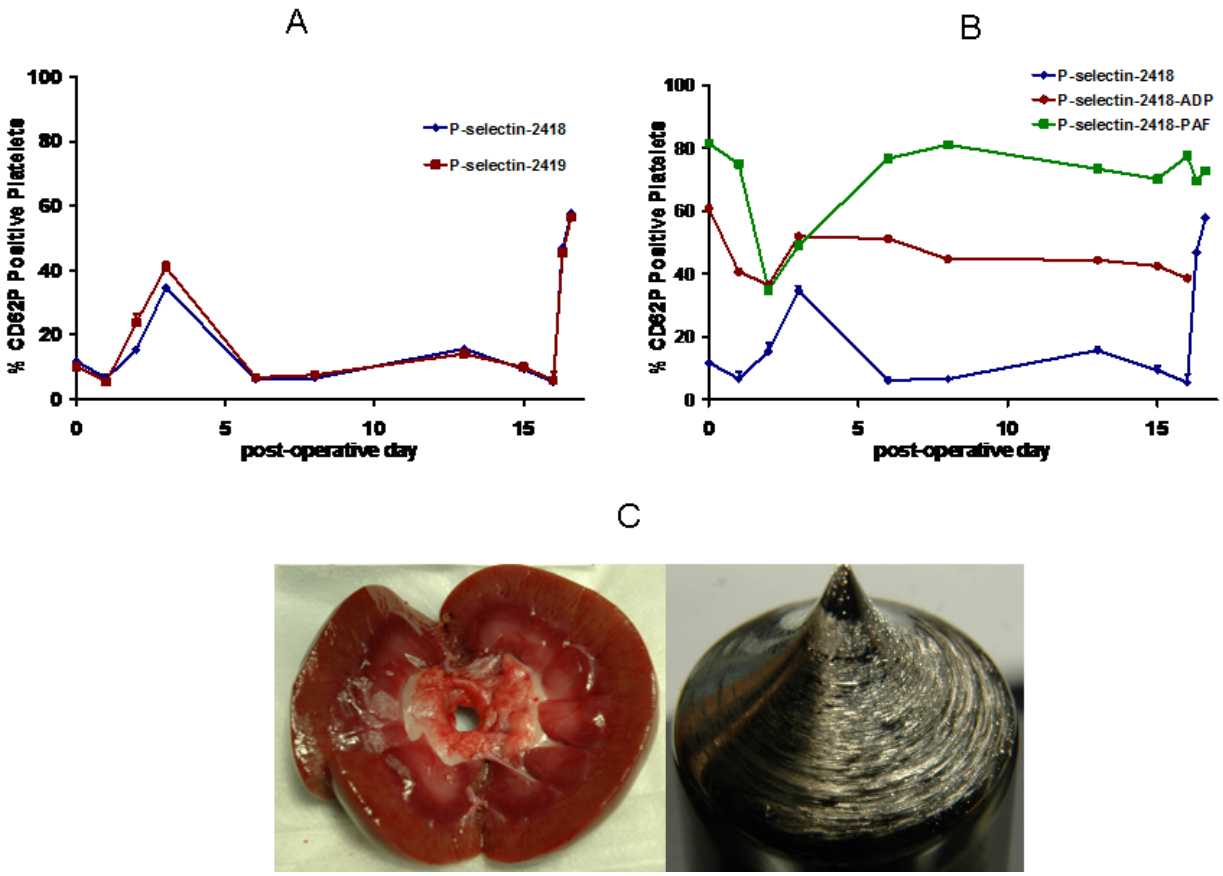

Figure 5-6 Platelet Activation and Necropsy Results from the first PF2 implant

A) Platelet activation in the first PF2 VAD chronic implant, B) platelet activation following stimulation of blood with 20 μM ADP and 10 μM PAF, and C) image of a kidney and the outflow region of the PediaFlow impeller at necropsy. Note: There is light reflection artifact on the left border of the kidney.

Platelet activation data and necropsy images for the 30-day PF2.1 VAD implant are seen in **Figure 5-7.** Platelet activation rose slightly following surgery reaching a maximum on day 1 and returned to baseline on day 2. The low platelet activation observed in this study was accompanied by an ability of platelets to become further activated following incubation with ADP and PAF. There were two small surface infarcts on the left kidney which did not penetrate past the cortex and a relatively low number of scratches on the impeller at necropsy.

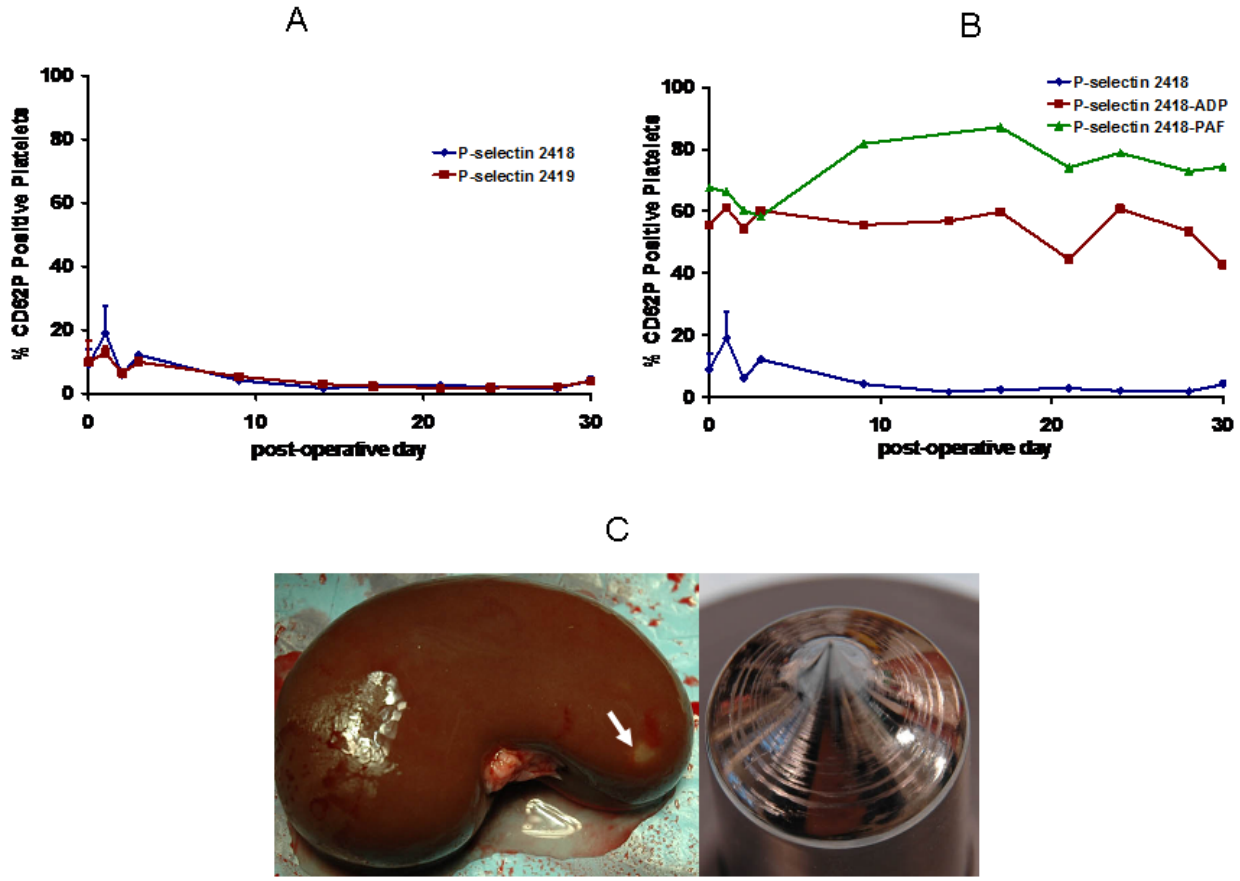

Figure 5-7 Platelet Activation and Necropsy Results from our second PF2 implant

A) Platelet activation in the second PF2 VAD chronic implant, B) platelet activation following stimulation of blood with 20 μM ADP and 10 μM PAF, and C) image of the left kidney and the outflow region of the PediaFlow impeller at necropsy. The arrow denotes the presence of a kidney infarct. Note: There is light reflection artifact on the left portion of the kidney.

Platelet activation and necropsy images for the 70-day PF2.2 VAD implant are displayed in **Figure 5-8**. Platelet activation in the 70 day study sharply rose following surgery reaching a maximum on day 3. On day 6 platelet activation began to decline, however between days 9 and 23 platelet activation measurements fluctuated with relatively high levels of activation detected by both antibodies on days 13 and 20. When platelet activation was high in the unstimulated samples the ability to respond further to ADP and PAF was limited. From day 23 until study termination on day 70, circulating activated platelet levels were relatively low, approaching baseline, and the platelets were highly responsive to agonist stimulation. At the time of device explantation several surface infarcts were observed on the left kidney; these infarcts did not penetrate into the medulla. There were also a moderate number of scratches found on the impeller of the device.

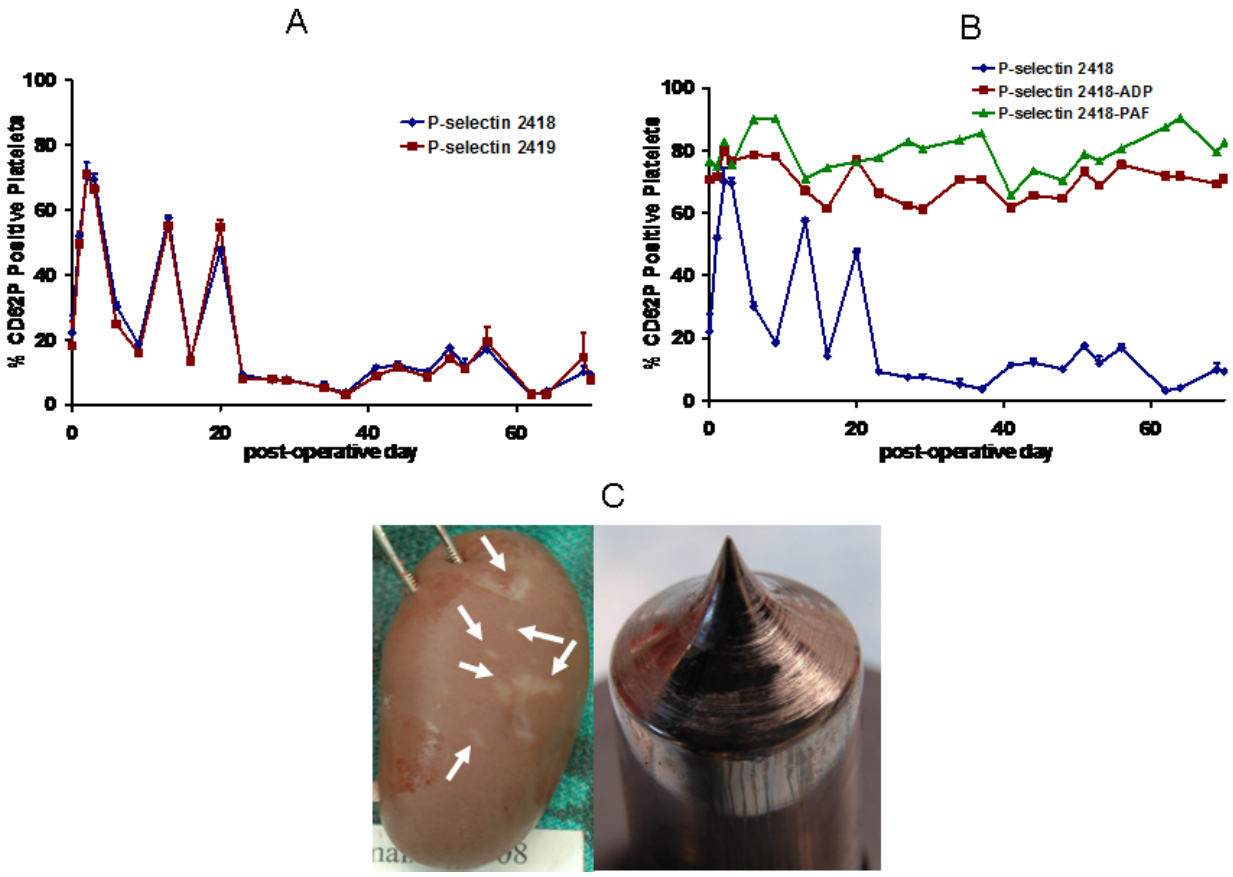

Figure 5-8 Platelet Activation and Necropsy Results from the third PF2 implant

A) Platelet activation in the PF2 VAD chronic implant, B) platelet activation following stimulation of blood with 20 μM ADP and 10 μM PAF, and C) image of the left kidney and the outflow region of the PediaFlow impeller at necropsy. Arrows denote the presence of kidney infarcts.

5.4 DISCUSSION

The recently available MCA2418 antibody was shown to cross-react and to differentiate agonist-stimulated from unstimulated ovine platelets, demonstrating its potential utility in quantifying platelet activation associated with blood contacting artificial organs. These results

are similar to results observed in **Figure 2-4** with MCA2419 and MCA2420. Each of these antibodies recognize human p-selectin on the surface of activated platelets, but being distinct clones they will recognize different epitopes on exposed p-selectin and may have differential binding to a given level of platelet activation. In assessing all of the data on these three antibodies with ovine platelets from **Chapters 2 and 4**, MCA2420 appears to have the lowest affinity for p-selectin relative to MCA2418 and MCA2419, and thus would be expected to be the least sensitive of the three platelet activation markers. Recently we have observed binding of MCA2420 to activated ovine platelets that was reduced compared to the initial data from **Chapter 2**. This reduced antibody binding persisted even with increased antibody concentrations. As a result, in future studies it is recommended to utilize two distinct antibody clones (MCA2418 and MCA2419) for control purposes and discontinue the use of MCA2420.

The in vitro study of the PF2 VAD in a blood perfusion circuit showed promising results with low platelet activation over the entire six hr study. The relatively low levels of platelet activation were accompanied by an ability to respond to PAF stimulus. The platelet response to PAF decreased over time and at the higher flow rates, but even at six hrs there was still a four-fold increase in the level of p-selectin positive platelets following stimulation. The PF2 results observed in vitro were similar to the low platelet activation observed in vivo, demonstrating the potential utility of the described platelet activation assays for in vitro testing. Measurements of platelet activation with an axial bearing pump in vitro showed elevations in the first hr to 18%. After stimulation however, platelet activation only rose to 22%. This minimal response to PAF suggested marked platelet dysfunction. The rise to 18% in the first hr of pumping by itself would not necessarily have been considered evidence of lack of platelet biocompatibility; however, the

inability of platelets to respond to stimulation suggests that substantial levels of activation and platelet damage have occurred.

This study is the first to apply these monoclonal antibodies for biocompatibility assessment in an in vitro mock circulatory loop using ovine blood. While limited to a single study, the contrast between the two pump types was dramatic and demonstrates the potential utility for more sophisticated in vitro studies. For example, this evaluation modality might be able to discern differences between similar pumps with different design parameters i.e. gap width, number of impeller blades, stator vanes; that would enable more focused research on the promising pumps in the more costly chronic in vivo studies.

Elevated pre-operative platelet activation has been observed in ovines as discussed in **Chapter 4**. In these reports stress was speculated to be a significant factor in this elevation and the stress was attributed to the necessity of animal restraint and jugular venipuncture to obtain blood samples. The time period for the sheep to acclimate to its new surroundings was also considered to be a potential factor. The use of an indwelling jugular vein catheter was applied in one animal in **Chapter 4**. In this report we performed a substantive comparison of platelet activation before and after placement of a jugular venous catheter. **Figure 5-3** shows pre-operative platelet activation was significantly decreased following placement of the jugular venous catheter, demonstrating the utility of the catheter in obtaining pre-operative platelet activation values. It is interesting to note though, that jugular venipuncture does not altogether preclude the acquisition of low pre-operative platelet activation as the spread in the data for venipuncture in **Figure 5-3** indicates. However, this large variability in platelet activation from

venipuncture samples provides further argument for the placement and use of a venous catheter. It is also important to note that clotting of blood samples collected via venipuncture was not uncommon. The use of a venous catheter is further merited because all post-operative samples are collected via an indwelling catheter thus ensuring a more appropriate comparison between pre-operative and post-operative values, in addition to avoiding comparison of post-operative data to artificially elevated baseline measurements.

VAD implantation generally requires invasive surgery (thoracotomy) that causes significant tissue damage, inflammation, and platelet activation. The effect of surgery on ovine platelet activation had not been previously quantified. Further, determining the extent to which surgery causes ovine platelet activation theoretically allows one to distinguish platelet activation that can be attributed to the VAD post-operatively. In this report platelet activation rose following the sham surgical procedure peaking on day 7 and then steadily dissipated over time back to baseline in approximately two and a half weeks. The ability of the platelets to respond to agonist similarly returned to baseline levels in this period. If temporal platelet activation after VAD implantation followed a trend similar to the temporal sham surgery it would indicate that minimal platelet activation was attributable to the device, a promising biocompatibility result.

Platelet activation in each of the PF2 VAD implants rose following surgery. In each case platelet activation returned to a baseline level. In the 17-day implant there was a large rise in activation on day 16 (**Figure 5-6**) which coincided with numerous pump stoppages due to a manufacturing defect in the axial position sensor cable in the percutaneous lead. With each pump stoppage there was rotor touchdown accompanied by regurgitant flow through the device

creating a flow field that may have promoted platelet activation. The numerous scratches found on the outflow section of the impeller (**Figure 5-6C**) may also have promoted activation. Despite the large spike in platelet activation it is worth noting that plasma free hemoglobin remained within normal limits. This large rise in platelet activation following pump stoppage was also observed in **Chapter 4**. In the 30-day implant of the PF2 VAD (**Figure 5-7**) platelet activation only rose to a modest degree (< 20%). There were several impeller scratches observed in this study, but the number of scratches appeared to be the least of the three implants. In the 70-day implant (**Figure 5-8**) platelet activation had the largest rise following surgery of the three implants but returned to baseline levels on day 9. However, in this study there were two subsequent sharp rises in platelet activation on days 13 and 20 although there were no observed pump stoppages or other pump complications on these days. At necropsy impeller scratches were identified and the cause of these scratches may have been related to the two spikes in platelet activation. Platelet activation did eventually moderate towards baseline on day 23 of this study.

Temporal platelet activation in ovines implanted with the PF2 VAD, particularly in the 17 and 30-day studies, showed similar trends to that of the sham studies in that there was a return to baseline values in the first two weeks. In fact, in these two studies platelet activation was lower than that observed in the sham surgery animals and platelet activation resolved more quickly. This is an encouraging biocompatibility result for the PF2 device, but merits some consideration as to how the VAD implanted animals would achieve such a result. In the sham studies the aorta had to be partially clamped in order to sew the outflow graft onto the aorta whereas with PF2 VAD placement the aortic cannula was inserted by a snare incision with the use of an introducer that required a much shorter aortic clamp time. In addition, coumadin was

not used in the sham surgery studies and while coumadin does not have a direct effect on platelets, the use of coumadin in the sham studies might have reduced thrombin generation and thus had an effect on the resolution of platelet activation after surgery. Platelet activation in the 70-day study was higher in the immediate post-operative period in comparison to the sham surgery control animals, which suggests that there was some ongoing activation directly attributable to the device. However, pump effects did resolve as platelet activation returned to a sustained baseline level on day 23.

In this chapter we also show more extensive results of platelet activation following stimulation with platelet agonists in the PF2 VAD-implanted ovines cohort. Low platelet activation levels would suggest good platelet biocompatibility however low platelet activation accompanied with an inability to respond to exogenous stimulation would suggest blunted platelet function as a result of irreversible pump-related damage or perhaps due to heavy anti-platelet medication. Assessing platelet responsiveness may be particularly useful in preclinical studies since a healthy animal would be expected to fully respond to platelet stimulus given that other pre-existing conditions (e.g. vascular disease, coagulopathies), prior surgery or device blood contact would not be present. The mostly low platelet activation measurements observed in the PF2 VAD-implanted ovines was accompanied by an ability of the platelets to respond strongly to exogenous ADP and PAF stimulation. At the end of the 17-day implant study and early in the 70-day study when platelet activation was high, there was a limited amount of further activation that was measured following stimulation.

Previous studies have related evidence of thromboembolism, as measured by kidney infarcts observed at necropsy, to levels of platelet activation measured to VAD implantation [63]. The initial level of platelet activation in the 70-day implant was higher than what was observed in the other PF2 studies and at necropsy there were more infarcts that were larger in size relative to the other two VAD-implanted ovines, although there was no evidence of kidney dysfunction during the implant. One of the reasons for the higher platelet activation and increased kidney infarcts may have been that the pump was operated at a much lower flow rate (0.5 L/min) than the first two implants. In the 17-day PF2.1 study (**Figure 5-6**) there was a large rise in activation at the end of the study without the presence of kidney infarcts. In this study the animal was terminated within 48 hrs of an increased frequency of pump stoppages and there may not have been enough time for a large thrombus to form or for a kidney infarct to develop grossly from a thromboembolus shed late in this period. A utility of flow cytometric assessment of platelet activation versus necropsy evaluation alone is demonstrated here in that it can provide an ongoing assessment of how the device is performing whereas it can be difficult to determine when an infarct has developed and how it might relate to pump or animal complications observed over the course of the study.

In comparing the results of these studies with the PF2 VAD to the PF1 results (**Chapter 4)**, some comments can be made. For the PF1 platelet activation became elevated at the end of the study or was sustained throughout the study for all three animals. In the PF2 series of implants all three had periods of sustained low platelet activation and only the initial 17-day study ended with elevated levels of platelet activation. Many of the complications encountered with the first generation PediaFlow implants were related to peripheral system failures (namely

cannula connections). This pump issue was eliminated in the PF2 VAD and the device underwent a motor redesign to achieve higher flow rates. Given the higher flow rates and higher associated shear the improved platelet biocompatibility observed in the PF2 VAD implants is encouraging. The PF2 VAD represents a promising next step in the PediaFlow development strategy to develop a pediatric VAD for infants and small children with high blood biocompatibility.

5.5 CONCLUSIONS

This chapter summarizes additional efforts taken to enhance blood biocompatibility assessment of cardiovascular devices in the ovine model. A new monoclonal antibody was characterized and demonstrated an ability to quantify circulating activated ovine platelets. The placement of a jugular venous catheter for blood collection was shown to significantly reduce pre-operative platelet activation artifact due to venipuncture. The effect of VAD placement surgery on ovine platelets was characterized and platelet activation attributable to the surgery was found to return to baseline levels in approximately two weeks and provides a means of a comparison for VADs implanted in the ovine model. Platelet activation following implant of the PF2 VAD also returned to baseline levels during the implant periods and platelets in contact with the PF2 VAD had preserved platelet function. The PF2 VAD demonstrated promising platelet biocompatibility and represents a critical next step in the development of a highly biocompatible PediaFlow pediatric VAD.

6.0 PLATELET ACTIVATION FOLLOWING IMPLANT OF THE THIRD GENERATION PEDIAFLOW DEVICE

6.1 INTRODUCTION

The PediaFlow Gen 2 devices showed very promising results with respect to platelet biocompatibility. In the PF2 series of implants there appeared to be improvement in the platelet activation results when compared to the PF1 device as the PF1 was plagued with numerous complications that impacted its biocompatibility results. Overall, the PF2 had improved flow characteristics and biocompatibility and the fundamental issue with PF2 was it was too big to be implanted in a newborn as seen in **Figure 6-1 (**courtesy of Dr. James Antaki**)**. In order to make the PediaFlow device small enough to be implantable in newborns a third generation (PF3) (**Figure 6-2,** courtesy of Launchpoint Technologies) device was developed which included a complete redesign of the fluid path, impeller blades (**Figure 6-3,** courtesy of Launchpoint Technologies, **compare to Figure 4-1, bottom panel**), stators, etc. These major design changes merit assessment of PF3 biocompatibility in terms of platelet activation. In this chapter platelet biocompatibility was characterized with both in vitro and in vivo studies of the PediaFlow PF3 device.

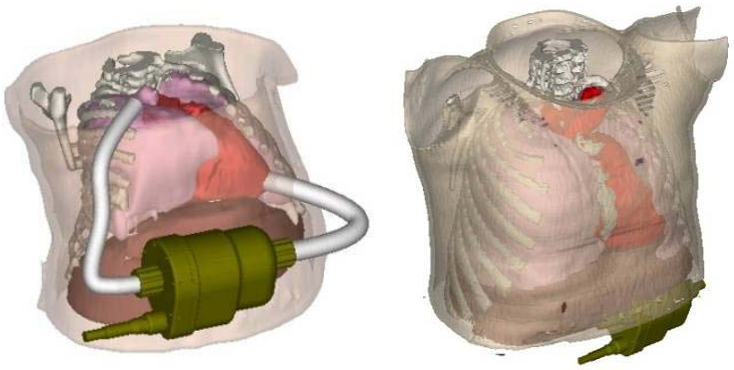

Figure 6-1: Image of the PF2 implanted in a 3 kg child (left) and in a 8 kg child (right)

Figure 6-2: The PediaFlow PF3 pediatric VAD

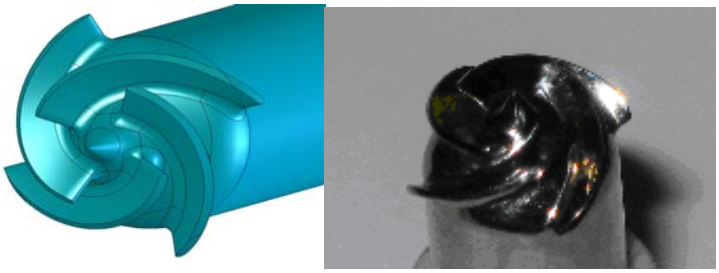

Figure 6-3 Computer generated and Digital PF3 Impeller Blade Topology Image

6.2 METHODS

In vitro biocompatibility flow loops

Mock circulatory test loops with a blood bag reservoir including the PF3 pediatric VAD was prepared as described in **Section 4.2**. In the PF3.1 study, the pump was added to the circuit and circulated for 2 hr each at 0.5, 1.0 L/min. There were concerns about rotor stability at higher flow rates. Following the first PF3 in vivo study a titanium ring was inserted to stabilize the rotor at higher flow rates; this updated PF3 is referred to as PF3.1b. The addition of this ring provided the necessary rotor stability to allow the pump to run at higher rpms. There were two additional in vitro studies performed with PF3.1b. These studies were performed for six hours duration at either 0.5 or 1.5 LPM. Blood was collected hourly for assessment of platelet activation in each in vitro study.

Assessment of Platelet Activation

Platelet activation samples were prepared as described in **Section 5.2**. Platelet activation was assessed during the in vitro studies and following stimulation with ADP and/or PAF. Platelet activation was assessed pre and post surgery with samples from each PF3 implant animal. In

addition, platelet activation was assessed following stimulation using ADP or PAF in samples from each of the PF3 implants.

In vivo studies

Two implants were performed with the PF3.1. Implant procedure and post-operative care was performed as described in **Section 4.2**.

6.3 RESULTS

Platelet activation results from the 4 hr in vitro test of PF3.1 are shown in **Figure 6-4**. Platelet activation during the PF3.1 in vivo study is depicted in (**Figure 6-5)** and after exogenous platelet stimulation (**Figure 6-6).** In this study the pump ran at a speed of 14.5 krpm, generating a flow of approximately 0.8LPM and lasted for 72 days, without any major complications.

Platelet activation results during two 6 hr in vitro tests of PF3.1b are shown in **Figure 6-7** (Pump speed: 0.5 LPM) and **Figure 6-8** (Pump speed: 1.5 LPM) respectively. Platelet activation is shown for the PF3.1b in vivo study before stimulation (**Figure 6-9**) and with stimulation (**Figure 6-10**). In the PF3.1b in vivo study the pump ran at 20 krpm for the first three days, providing a flow of 1.1 LPM. Beginning on day three the study was compromised with numerous rotor touchdown events. The speed was eventually turned down to 16.5 krpm and generating a flow of approximately 0.8 LPM. On postoperative day 9 the pump could not be restarted and the study was terminated. The electrical connection to the axial position sensor was damaged and was concluded to be the cause of the rotor touchdown events. **Figure 6-11**

illustrates platelet activation from the PF3.1b study including samples after rotor touchdown had occurred.

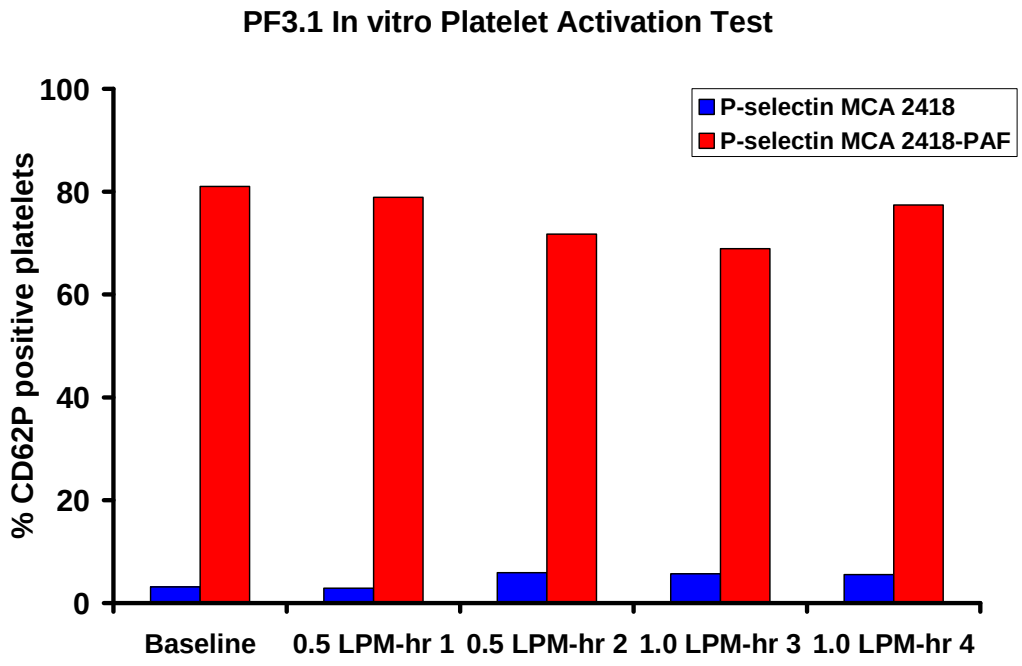

Figure 6-4 PF3.1 platelet activation in vitro test

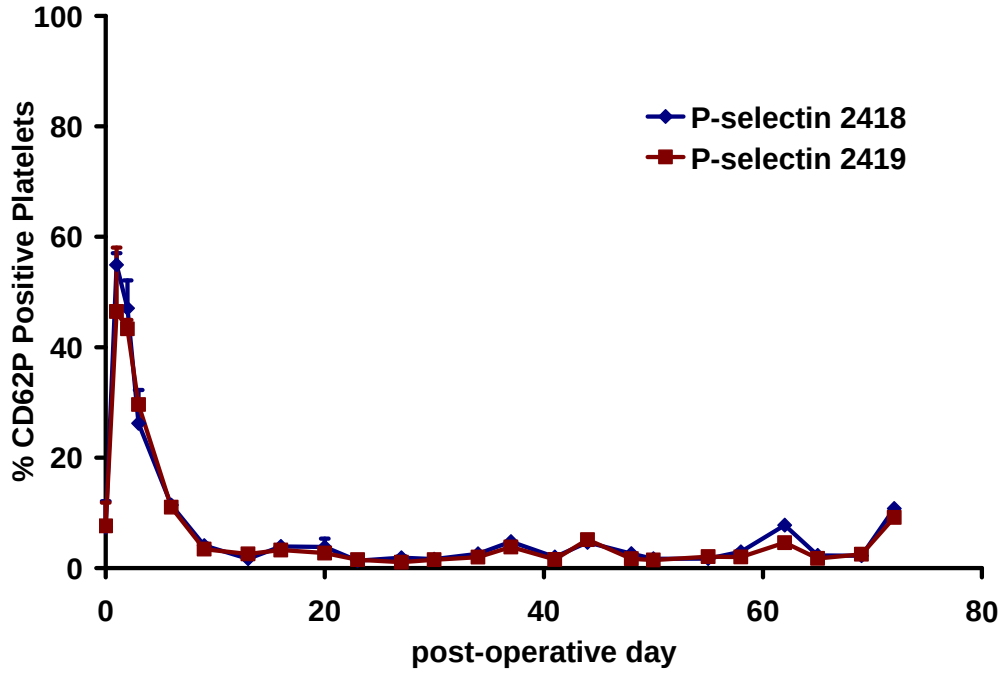

Figure 6-5 Platelet activation of the PF3.1 implant

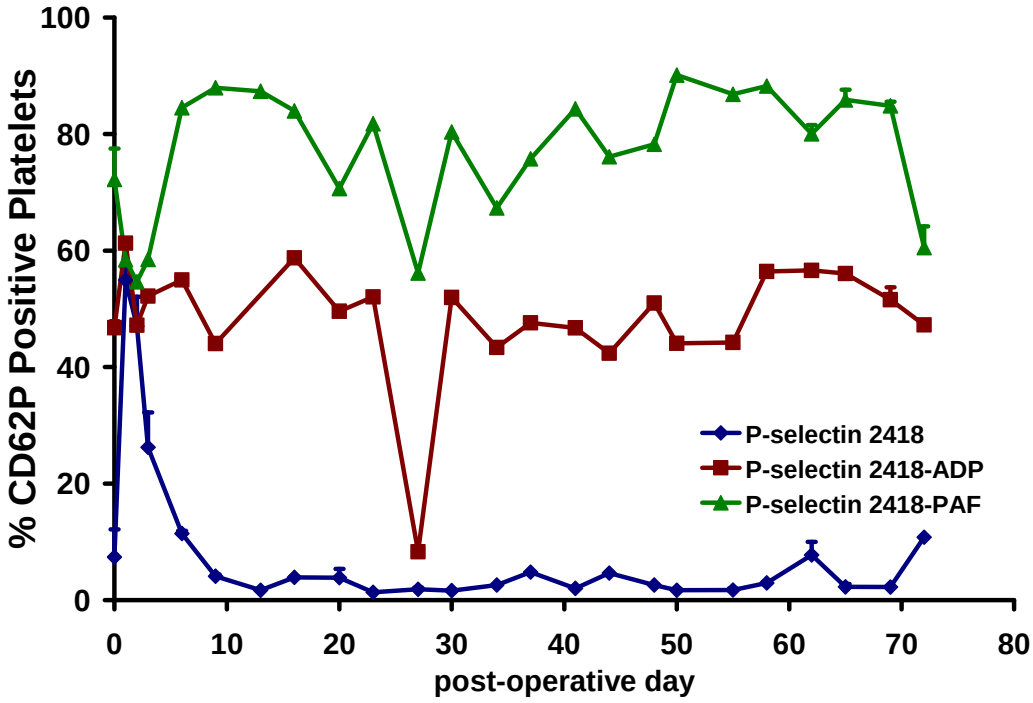

Figure 6-6: Platelet activation following PF3.1 implant with stimulation

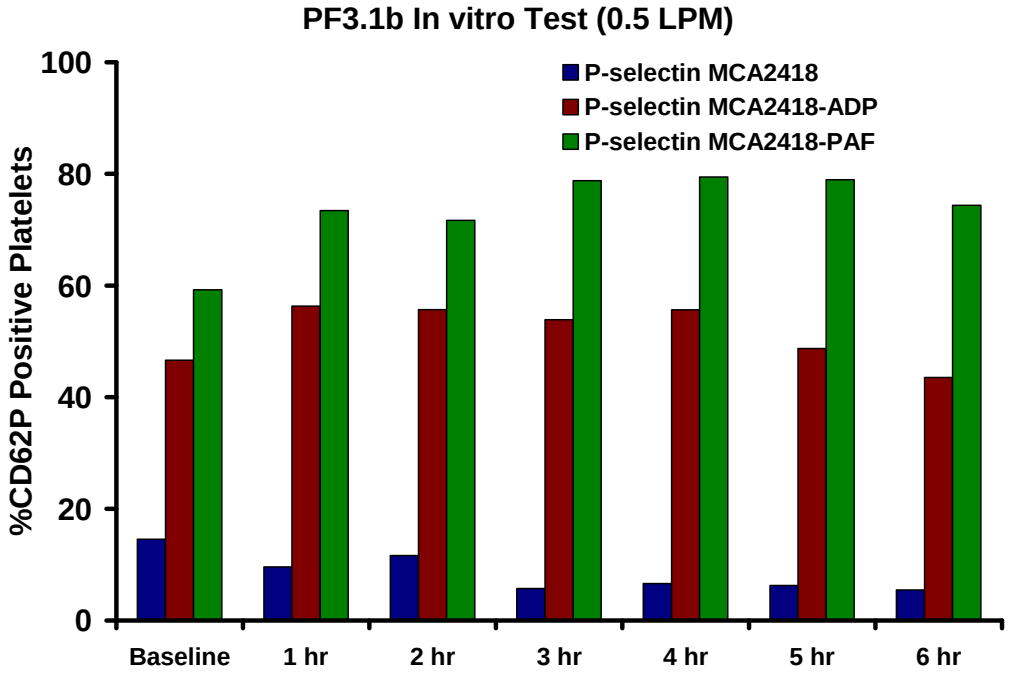

Figure 6-7: PF3.1b in vitro platelet activation study (0.5LPM)

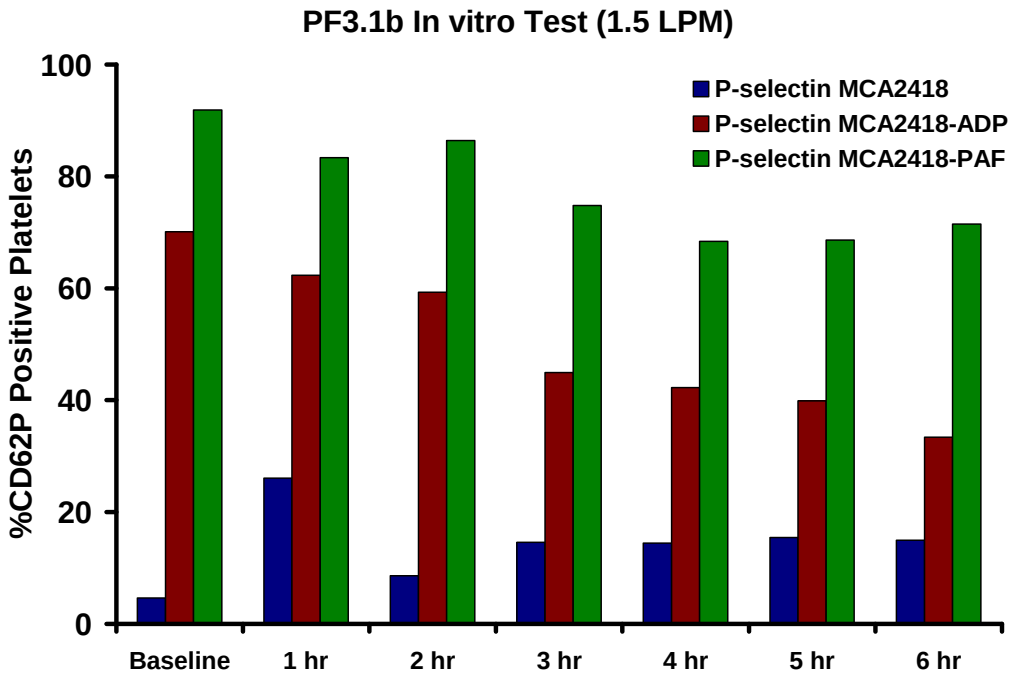

Figure 6-8: PF3.1b in vitro platelet activation study (1.5LPM)

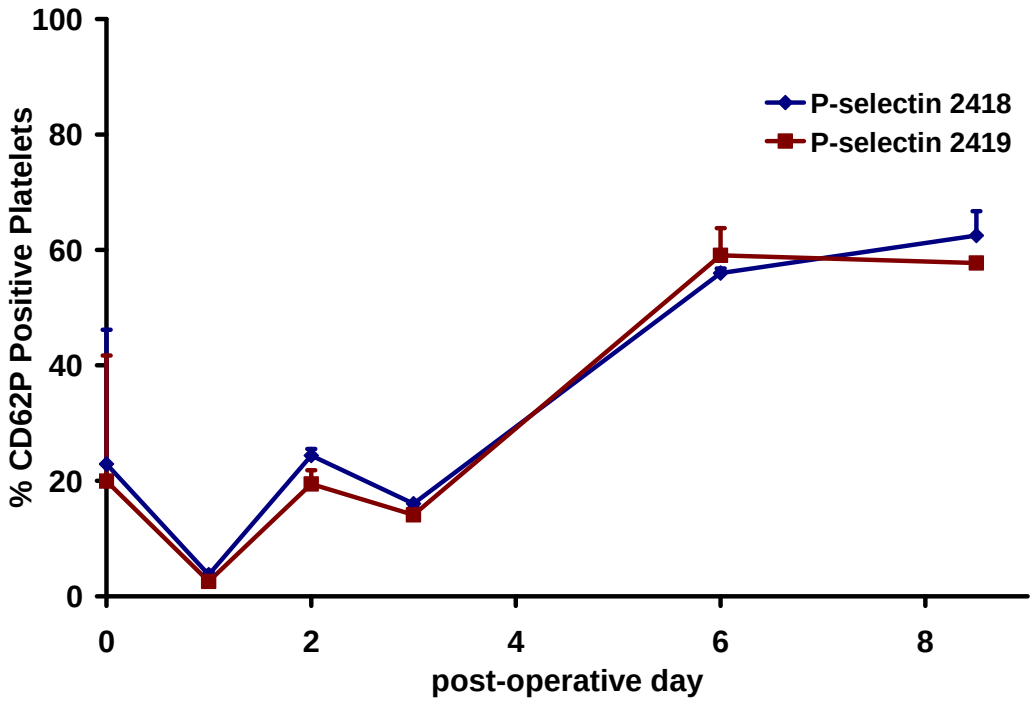

Figure 6-9: Platelet activation following PF3.1b implant

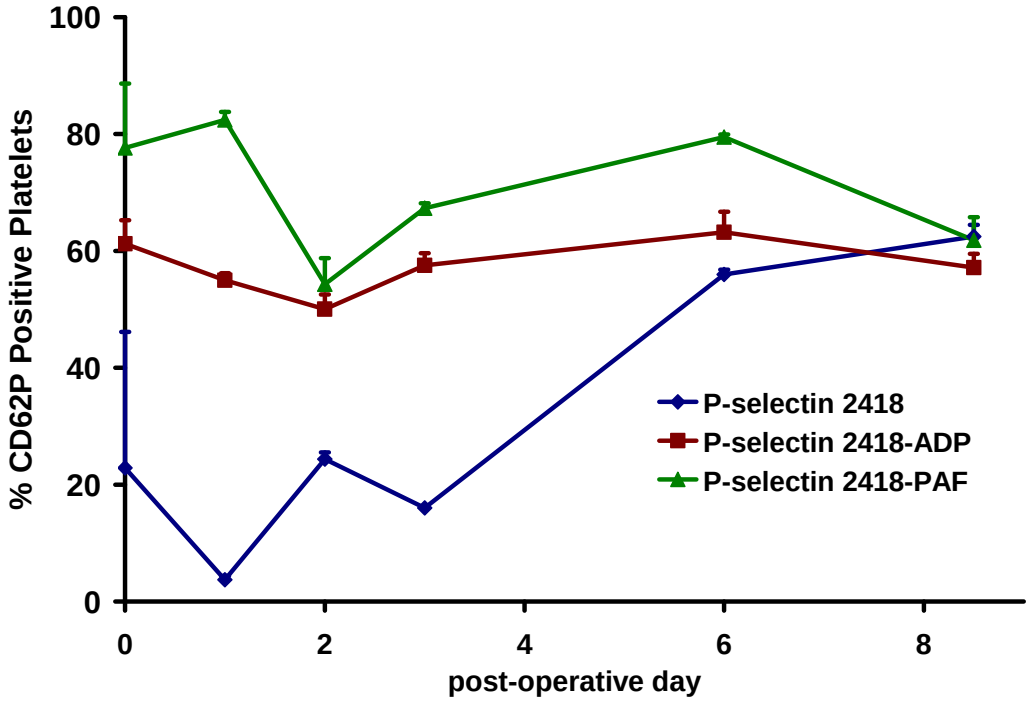

Figure 6-10: Platelet activation following PF3.1b implant with agonist stimulation

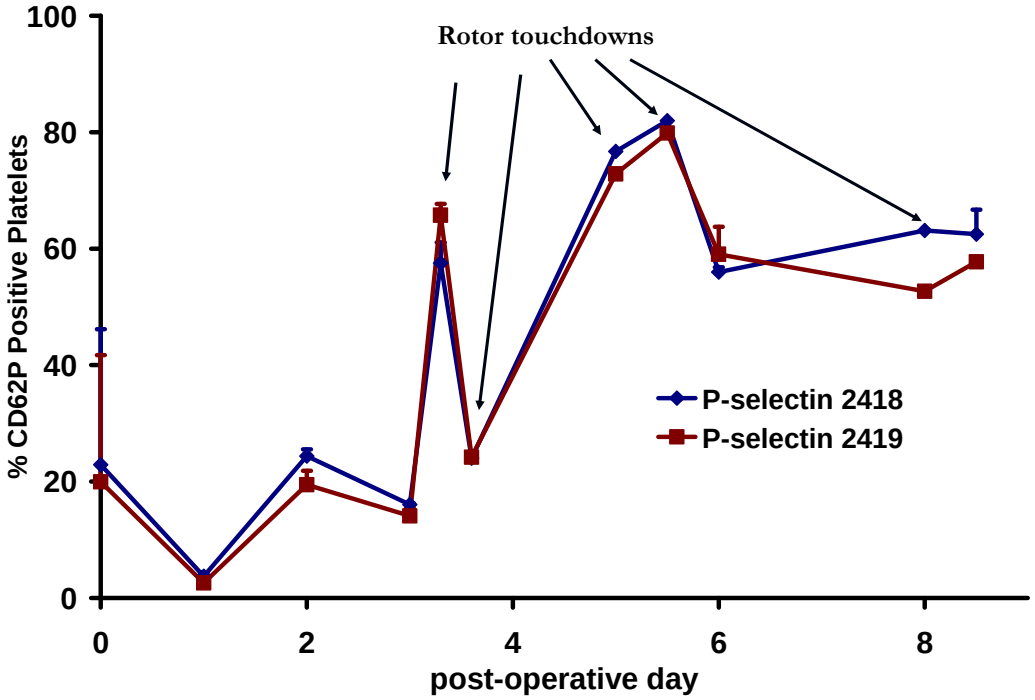

Figure 6-11 Platelet activation of the PF3.1b including samples after rotor touchdown occurred.

6.4 DISCUSSION

Following implant of the PF3.1 platelet activation sharply rose and by day 9 had returned to baseline. This temporal response was comparable to what was observed in the sham surgical studies (**Figure 5-5A**). In fact platelet activation was back to baseline more quickly than what was observed in the sham studies. This phenomenon was also observed in the PF2 studies, and attributed to the longer aortic clamp time necessary in the sham studies and the lack of coumadin used in the sham studies. However, in the PF3 studies a vascular graft was sewed onto the aorta,

and as a result the PF3.1 implant was very similar to the sham surgeries with the exception of the use of coumadin.

Following surgery platelet response to agonist did diminish while the platelets were highly activated prior to in vitro stimulation, this post-surgical response has been observed in other studies. When platelet activation returned to baseline, platelet response to stimulation also increased. On day 27 of the study however platelet activation following stimulation with ADP and PAF was reduced. Since platelet activation after stimulation with both ADP and PAF were reduced, it is reasonable to project that there was some platelet dysfunction occurring at this data point. It is also important to note that this platelet dysfunction was not sustained as platelet activation after stimulation with ADP and PAF was back to its baseline levels on day 30 (the next data point). The response to ADP was so low in fact that it appeared that no agonist had been added. In future studies we endeavor to perform the stimulation studies in duplicate so as to diminish the possibility of user error. On day 70, there was also a drop in platelet response to PAF, but because platelet response to ADP was unchanged, it was concluded that there was no change in platelet function. Since ADP is a weaker activator of platelets, it is thought that a decrease in platelet function would first be observed as a reduction in platelet response to ADP.

In the sterilization process the housing for the PF3.1b came off and it was not possible to securely fasten on the pump housing during the study. It is possible some fluid may have been entrapped in the housing. The loose housing resulted in a damaged pin connecting the housing to the axial position sensor. This damage to the axial position sensor caused numerous pump touchdown events, which resulted altered flow (regurgitation). The numerous pump stoppages

and subsequent restarts also likely contributed to embolization of thrombus as evidenced by a number of infarcts at necropsy. These events certainly impacted platelet activation and from day 3 to day 6 there was a sharp rise in the level of platelet activation through the end of the study. Platelets responded to agonist stimulation through day 3, but after day 3 platelets were already highly activated and there was only a limited response to agonist above the already elevated platelet activation levels. In **Figure 6-11** platelet activation is shown for the PF3.1b study including samples taken when rotor touchdown events were known to have occurred. Platelet activation in samples following rotor touchdown events were typically elevated above platelet activation in samples where touchdown events were not known to occur.

The environment in the pump following numerous pump stoppages is a clear nidus for platelet activation. In PF1 (**Figure 4-7C-D)** and in PF2 (**Figure 5-6 A-B)** implants where pump stoppages were known to occur there were sharp rises in platelet activation. This underscores again the utility of the platelet activation assays in that they consistently show a response when there are known pump complications. Given the positive results observed with the initial 3.1 study it is certainly possible that were it not for the issues with the axial position sensor, a major nidus for platelet activation, the PF3.1b study could have exhibited more promising platelet biocompatibility. At necropsy there was no thrombus observed around the titanium ring that was placed to stabilize the rotor, the major difference between PF3.1 and PF3.1b, so it is unlikely that the ring was a nidus for platelet activation.

6.5 CONCLUSIONS

The PF3 device with its size close to that of an AA battery is now small enough to be implanted into newborns. Platelet activation in each of the in vitro flow loops showed promising low levels of activation coupled with a robust response to exogenous stimulation. Platelet biocompatibility in the initial study was very promising with a return to baseline platelet activation within 9 days, which represented a swifter resolution of platelet activation than the sham studies. Platelet activation following exogenous stimulation was appropriate, signifying retained platelet function. In the second chronic study, platelet activation was highly sensitive to the numerous rotor touchdown events again illustrating its utility in the assessment of pump biocompatibility. Further efforts to increase the flow of this pump while securing the axial position sensor are needed. Along with the overall promising platelet activation results, the miniaturization of the PediaFlow in this third generation device is a critical step in the goal to yield an implantable, highly biocompatible blood pump for newborns.

7.0 PLATELET ACTIVATION FOLLOWING IMPLANT OF THE LEVITRONIX® PEDIVAS™

7.1 INTRODUCTION

The Levitronix® PediVAS™ (**Figure 7-1,** courtesy of Dr. Kurt Dasse) is a magnetically levitated extracorporeal pump leveraged from the Levitronix CentriMag, an adult pump that has been implanted in over 6000 patients worldwide (personal communication-Levitronix). The CentriMag has been successfully applied as a bridge to decision as an LVAD, RVAD, or BiVAD and further has been used as the pump head in ECMO circuits [126-129]. The PediVAS can provide 0.3 to 3.0 liters of flow for up to 30 days duration and has been implanted in over 500 children worldwide [23]. There is a need for such devices able to provide short term support as a bridge to decision to a longer term support device, or to allow the myocardium time to recover [23]. The ability of the PediVAS to generate "lower" flow rates (< 2 LPM) is important as it is estimated that the largest cohort of pediatric patients that would benefit from ventricular assist devices (VADs) are less than one year of age [20, 22] . The PediVAS is currently undergoing final preclinical studies in anticipation of beginning a clinical trial in the United States. The objective of this chapter was to further characterize the blood biocompatibility of the Levitronix PediVAS in a cohort of studies in terms of circulating activated platelets using commercially available cannula and customized Levitronix cannulae (**Figure 7-2**, courtesy of Dr. Kurt Dasse).

7.2 INITIAL CHRONIC PEDIVAS STUDIES

Figure 7-1: Levitronix PediVAS system, and close up of the PediVAS pump head.

7.2.1 Methods

Implant procedure

We employed lambs weighing approximately 20–30 kg to test the performance and biocompatibility of the PediVAS. Surgical procedure and post-operative care were performed as in **Section 5.2** with the following alterations. The pump and extracorporeal circuit were primed with warm balanced electrolyte solution, connected to the cannulae, and the system was placed adjacent to the animal to minimize extracorporeal tubing (medical grade Tygon) lengths. An ultrasonic flow probe was connected onto the outflow tubing. The pump was started and

operated at the following flow range: 0.5-1.5 LPM. The animal was allowed to awaken immediately after surgery and supported with a vest and sling restraining system.

Figure 7-2: Levitronix custom cannulae

Assessment of cellular activation

Preoperative whole blood was collected from healthy ovines by jugular venipuncture. Post-operative blood was collected as in **Section 4.2.** Platelet microaggregates were measured as described in **Section 2.1.2**. Platelets binding Annexin V was measured as described in **Section 2.3.2**. Leukocyte platelet aggregate tubes were prepared with 120 μL of the Tyrode's buffer, 5 μL of 75 μg/mL GB20A (ovine platelet marker, VMRD) or 5 μL of 75 μg/mL Coli S69A, 100

μL of blood, and 5 μL of 300 μg/ml goat anti-mouse phycoerythrin (Invitrogen, Carlsbad, CA) and incubated for 20 minutes. Samples were then washed and resuspended as previously described. Anti-ovine CD45 (pan leukocyte marker; 10 μL; AbD Serotec) was added and incubated for 20 minutes. ACK buffer was added to lyse the RBCs and the samples were centrifuged and resuspended as before, washed with Tyrode's buffer with citrate, then fixed with 1% paraformaldehyde. A 2% fluorescent intensity threshold was set based on the isotype control antibody to leukocytes (CD45-positive cells); leukocytes with GB20A fluorescence above this threshold were considered leukocyte platelet aggregates.

7.2.2 Results

Measures of platelet activation by flow cytometry including Annexin V, platelet microaggregates, and platelet leukocyte aggregates are plotted in **Figure 7-3**. All of the platelet activation indices all increased slightly after the implant surgery. Annexin V increased markedly on day 14 and remained elevated through day 28. All of the indices returned to below or slightly elevated levels compared with the preoperative values by the conclusion of the study.

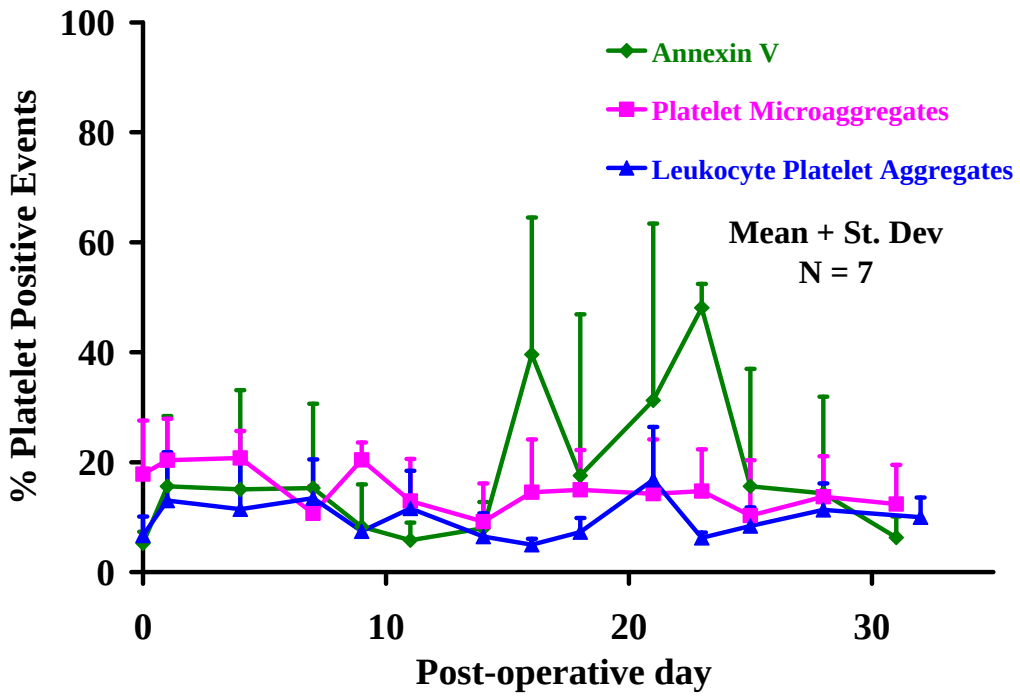

Figure 7-3: Platelet Positive Events following initial PediVAS implants

Flow cytometric assays of platelet activation. Assays of Annexin V, platelet microaggregates, platelet leukocyte aggregates, were obtained during a 30-day period of observation after device implantation. These indices increased during the implant period but returned to levels below or slightly elevated compared with the preoperative levels.

7.2.3 Discussion

Initial studies with the Levitronix PediVAS produced positive hemodynamic results in generating the desired flow rates of 0.5 – 1.5 LPM. However the limited experience with the ovine model in these initial studies led to a number of issues with the implant procedure, anesthesia, anticoagulation, as well as respiratory management. A number of the assays developed in **Chapter 2** were not yet available for biocompatibility assessment. There was very little increase in the indices following surgery and in the case of leukocyte platelet aggregates

and platelet microaggregates changed very little over the course of the study. The validity of the leukocyte platelet aggregates assay as discussed in **Section 3.2.3** remains a question here until the utility of the assay can be confirmed through cellular visualization, or other means. Annexin V did sharply rise after 14 days suggesting some ongoing platelet activation; however, this activation was not sustained. As mentioned above these initial studies were ongoing along with much of the assay development outlined in **Chapter 2**. The Annexin V assay must be analyzed on the flow cytometer right after the incubation period because there is calcium in the Annexin V binding buffer used in the assay which can activate the platelets if the blood is not analyzed soon after the incubation period. As a result it is possible that inexperience with this assay may have led to some of the elevated Annexin V binding to platelets after the initial two weeks. This hypothesis is feasible since in subsequent PediVAS and PediaFlow in vivo studies using the Annexin V assay, its binding to platelets typically was not elevated even when there was substantial p-selectin expression on platelets, thrombotic deposition in the cannula or evidence of kidney infarcts. It is important to note that Annexin V binding did return to baseline by the end of the study. If the increase in Annexin V was strictly due to the PediVAS pump, it is encouraging that pump-induced activation dissipated but not as promising as sustained baseline levels after the initial surgical insult.

7.3 TEMPORAL PEDIVAS STUDIES APPLYING DEVELOPED FLOW CYTOMETRIC P-SELECTIN ASSAYS

7.3.1 Methods

Blood collection

Pre-operative blood was drawn via jugular venipuncture using an 18-gauge 1.5-in needle with syringe, discarding the first 3 mL. Blood (2.7 mL) was then added to sodium citrate tubes. At least two pre-operative samples were collected and averaged to obtain the pre-operative data point (post-operative day zero). Post-operative samples were collected as described in **Section 4.2.**

Assessment of platelet activation

Platelet activation was assessed pre and post surgery with samples from each animal implanted with the Levitronix PediVAS. In animals implanted with the PediVAS and its custom cannula platelet activation was also assessed following stimulation using with PAF. Samples for platelet activation assessment were prepared as described in **Section 5.2**.

In vitro biocompatibility flow loop setup

A mock circulatory test loop with a blood bag reservoir and the Levitronix PediVAS was prepared as previously described in **Section 5.2**. Ovine whole blood was collected by jugular venipuncture into a blood bag with of 0.106M sodium citrate solution (1 to 10 by volume). Ovine blood (90 mL) was added to the reservoir and circulated for 1 hr at 1.65 L/min against a pressure

of 100 mm Hg. Baseline and 1 hr blood samples were collected and assessed for platelet activation (before and after stimulation with PAF).

Implant procedure

Healthy ovines (13; 20-30 kg) were implanted with the Levitronix PediVAS. Implant procedure and post operative was completed as described in above in **Section 7.2.1.** The inflow cannula used was either commercially available **(**cardiopulmonary bypass cannula (Medtronic, DLP) or custom Levitronix inflow cannula **(Figure 7-2)** and the outflow cannula used was either outflow graft (Vascutek) or the custom Levitronix outflow cannula.

7.3.2 Results

During the one hour in vitro study baseline platelet activation was 8.6% and following stimulation rose to 72.5%. After one hour of blood contact in the platelet activation was 2.3% and after PAF stimulation was 69.2%.

In vivo PediVAS studies using commercially available cannulae

Figure 7-4 shows platelet activation following implants of the PediVAS system with commercially available cannula that had uncomplicated post-operative courses. Platelet activation rises following surgery and by thirty days has returned to baseline. **Figure 7-5** shows platelet activation following implants of the PediVAS with commercially available cannula in the subset of studies that had post-operative complications including air embolus and thrombotic cannula occlusion. Platelet activation in these cases rise following surgery and begin to decrease but following complications platelet activation rises and remains highly elevated at day 30. In the

30 day study where an air embolus occurred on day 23 platelet activation returned to baseline at day 10; and the average platelet activation from day 10-21 was 9.5% After the air embolus occurred (day 23), platelet activation sharply rose and the average level was 59.1% from day 23-30.

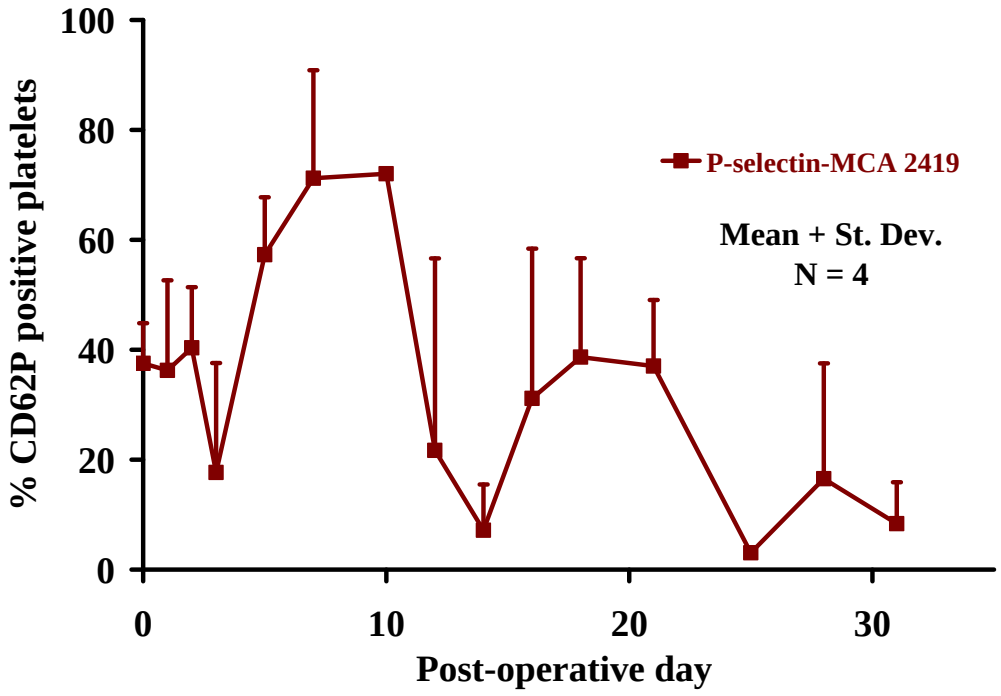

Figure 7-4: Platelet activation following Levitronix PediVAS implant using commercially available cannula without post-operative complications.

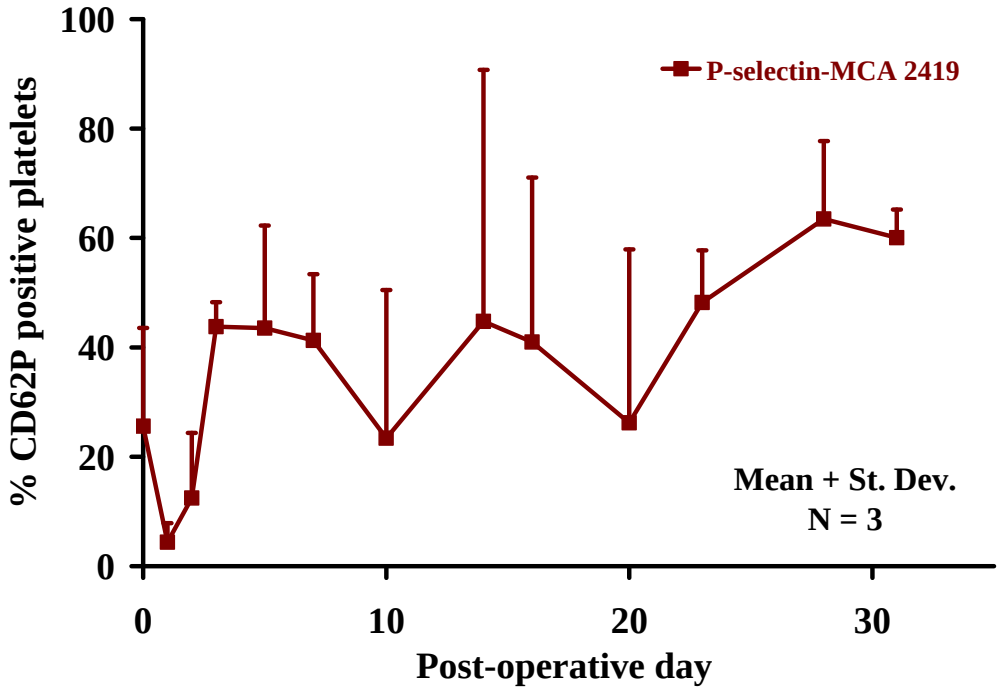

Figure 7-5: Platelet activation following Levitronix PediVAS implants using commercially available cannula which had post-operative complications

In vivo PediVAS studies using custom cannulae

Platelet activation following implant of the Levitronix PediVAS using the custom cannulae in the subset of animals that had few or no kidney infarcts is depicted in **Figure 7-6**. Platelet activation in these animals rose following surgery but came back down to below baseline values. Platelet activation following stimulation was substantially higher than platelet activation prior to stimulation. Platelet activation in the subset of animals that had mild to moderate kidney infarcts and/or thrombus deposition in the cannulae are illustrated in **Figure 7-7**. Platelet activation in these animals rose following surgery and remained elevated throughout the surgery. In these studies platelets demonstrated a limited ability to further respond to exogenous platelet stimulus. In one of these studies where the implanted animal had a moderate number of kidney

infarcts and thrombotic obstruction of the inflow cannula platelet activation was elevated at 50.4% on day 27 and following PAF stimulation was 50.9%, on day 30 platelet activation dropped to 18.7% and following PAF stimulation was 14.9%.

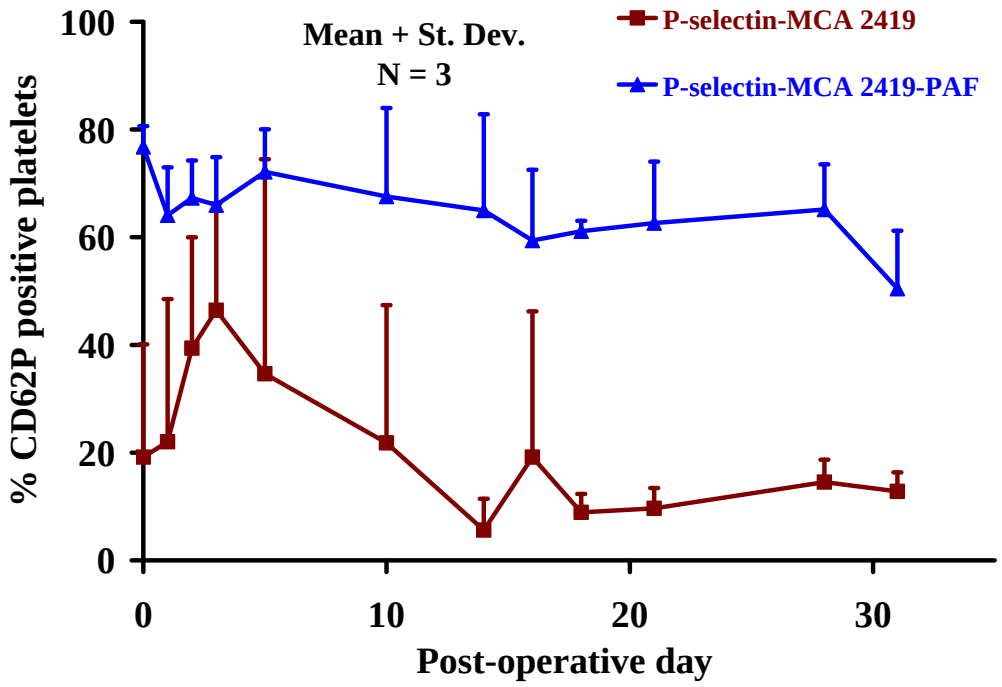

Figure 7-6: Platelet activation following Levitronix PediVAS implant using the customized cannula before and after stimulation with 10 μM PAF in implants that had few/no kidney infarcts.

7.3.3 Discussion

The Levitronix PediVAS was designed without bearings, seals, and with large fluid gaps to promote biocompatibility [23-24]. The CFD results from Zhang et al also showed no areas of stasis and in the majority of the flow field did not have areas of high shear (< 100 Pa) which suggested the potential for promising biocompatibility [24]. In fact, promising hemolysis levels were demonstrated in vitro and in vivo [23-24]. In this report, the acute in vitro study

demonstrated that platelet activation did not increase from baseline and platelets retained their ability to respond to stimulus, signifying the potential for good platelet biocompatibility.

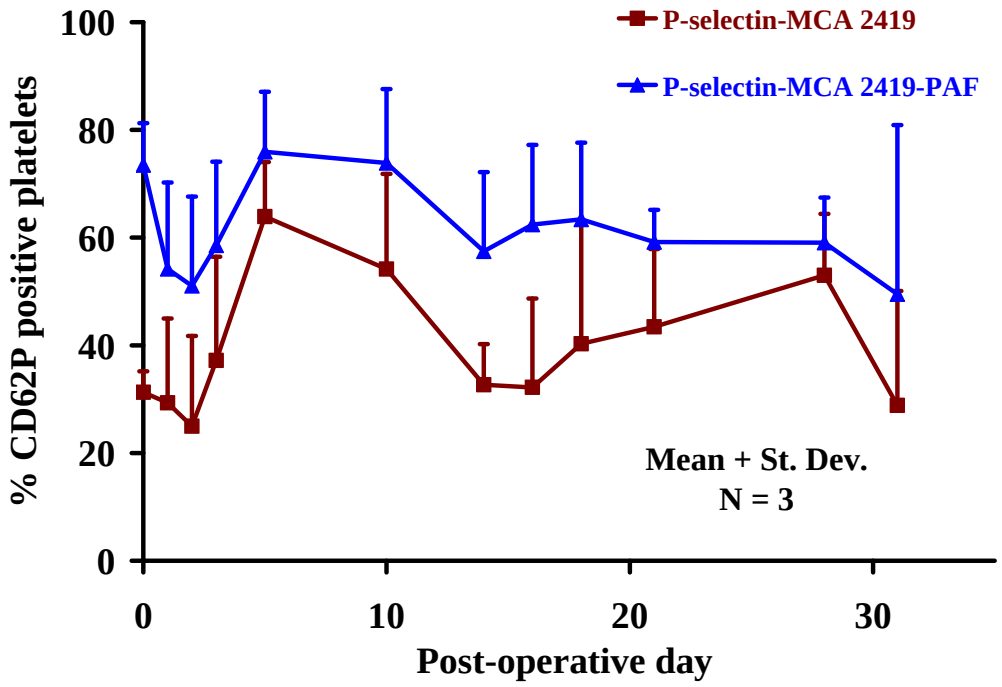

Figure 7-7: Platelet activation following Levitronix PediVAS implant using the customized cannula before and after stimulation with 10 μM PAF in implants that had a moderate number of kidney infarcts and/or thrombus deposition in the cannulae

High pre-operative platelet activation was an observation in **Sections 4.4** and **5.4** and has been attributed to animal stress during blood collection. In PediVAS implants however we were not able to place jugular venous catheters pre-operatively in this study as in **Sections 4.4** and **5.4**. Platelet activation following implant of the PediVAS (**Figure 7-4 to Figure 7-7**) was elevated in the early post-operative period as expected following surgery. As a result, the high pre-operative platelet activation makes it more challenging to assess post-operative platelet activation as it appears that platelet activation does not rise following surgery. Without the pre-operative artifact however, a post-operative rise following surgery would be appreciated.

In the ovine sham surgical studies platelet activation (**Figure 5-5**) due to the surgical insult rose following surgery and returned to baseline in approximately two weeks. Persistent platelet activation as seen in **Figure 7-4, Figure 7-5, & Figure 7-7** suggests ongoing platelet activation past the initial two weeks. Since the implanted animals were otherwise healthy, ongoing platelet activation after two weeks can be attributed to the PediVAS device. Since the PediVAS is an extracorporeal device it is also important to note that deployment of this device requires the use of a variable length of medical grade Tygon tubing along with connectors that may impact biocompatibility. Given the surface area and length of Tygon tubing in contact with blood, its impact on biocompatibility may not be negligible. Platelet activation in the majority of the studies (**Figure 7-4 and Figure 7-6**) however, did return to baseline by the conclusion of the study which is an encouraging result.

Several of the PediVAS implants did however have some complications and in these studies platelet activation (**Figure 7-5**) remained elevated. The complications included air embolus and thrombus deposition on the Tygon tubing or in the inflow cannula. The air embolus occurred on day 23 of one of the studies and resulted in a sharp rise in platelet activation following the occurrence. The air embolus occurred as a result of a human error while collecting a blood sample from the arterial line and was not the result of cavitation or other pump related issue. In this study there was a promising platelet activation time course before the embolus, with a return to baseline by the tenth day, mirroring the temporal course of the ovine sham studies (**Figure 5-5)**. This particular 30 day study illustrated the sensitivity of the platelet activation assays as platelet activation responded to the observed animal complication.

Cannula design is an essential component of VAD development and cannula design parameters (shape, material, etc.) can have a significant impact on hemodynamic performance and biocompatibility [130-131]. As a result it is essential to evaluate the hemodynamic and biocompatibility performance of the PediVAS along with the customized cannula. Platelet activation results with the custom cannula are displayed in **Figure 7-6 & Figure 7-7**. In **Figure 7-6**) platelet activation returned to below the elevated baseline levels and followed a temporal course similar to that observed in sham surgical controls (**Figure 5-5)**. Platelets from these studies responded to agonist stimulus throughout the time course signifying that platelets following blood contact with the PediVAS retained their functionality. The low observed platelet activation was accompanied with few or no kidney infarcts observed at necropsy.

In the custom cannula cohort where there were moderate number of kidney infarcts and/or thrombotic deposition of either the inflow or outflow cannula at necropsy there was a higher level of platelet activation (**Figure 7-7**) compared to the "few/no infarct" group (**Figure 7-6)** . There was a lesser amount of further activation possible in the moderate infarct group due to the higher initial platelet activation. These results suggest a potential relationship between the level of platelet activation and the level of kidney infarcts and signify the utility of the platelet activation assays employed in this study.

In one of the studies from the moderate infarct group platelet activation dropped from 50.9% to 18.7% on the last day. This would have been considered a positive result however platelet activation following stimulation also drops markedly to 15%. Platelet activation

following stimulation would have been expected to remain elevated; its descent suggests marked platelet dysfunction at the end of this study. This study illustrates the importance of employing stimulation assays during biocompatibility studies as the low final platelet activation result on the final of this singular study would have given the impression of improving biocompatibility when biocompatibility was diminished. At necropsy there were several cortical infarcts present, and large thrombi lodged in both the inflow and outflow cannulae. Despite nearly total occlusion of the outflow and inflow cannula however, it is worth noting that serum chemistry values remained within normal limits.

In the other two implants from the custom cannula cohort with moderate kidney infarcts, serum chemistry values (creatinine) for kidney function were normal, which indicates that despite some thrombotic injury to the kidney there was no permanent renal damage. Preservation of normal kidney function is a positive result but perhaps underscores the limitations of renal serum chemistry values in the biocompatibility assessment of artificial organs. Creatinine however is thought to be a poor indicator of kidney injury [132]. Severe kidney damage would be necessary before these values became abnormal. More sensitive markers of acute kidney injury are becoming available including: liver-type fatty acid binding protein, serum or urine levels of neutrophil gelatinase associated-lipocalin, urinary levels of kidney injury molecule-1, and urinary levels of IL-18 [133-135]. While it is promising that platelet activation assays used in this chapter appear to bear promise in detecting subclinical thrombotic damage it would be interesting to note if these more kidney injury markers become elevated in sheep that have numerous kidney infarcts and if so could be compared to platelet activation results as a means to further discriminate the sensitivity of the employed platelet activation assays.

It is unknown exactly what level of platelet activation and kidney infarcts would preclude a device preceding to a clinical trial, but clearly the least amount of platelet activation and kidney infarcts that a pump design generates would be desirable, as this would ensure the most biocompatible device is sent for clinical use. However, it may be unlikely to expect that a pump will generate minimal platelet activation in preclinical studies. Preclinical studies of the HeartMate II VAD implanted in the bovine model showed that platelet activation was persistently elevated (35-55%) after the surgical effects had dissipated [63]. In the clinic the HeartMate II has been approved by the FDA and has been successfully implanted in thousands of patients; however, stroke rates have been reported to be as high as 18%, not including other cases of device thrombosis [136-138]. While the HeartMate II has been immensely successful, the stroke rates can still be improved. The promising clinical results with the HeartMate II despite persistent platelet activation in preclinical animal models suggest that further reduction in observed preclinical platelet activation after the effects of surgery have dissipated could potentially lead to lower stroke rates in clinical practice and this should be the focus during preclinical VAD design. A level of 20% or less platelet activation with preserved platelet function after the effects of surgery have dissipated would appear to be a good design goal to target. In our experience studies where ovine platelet activation was less than 20% after surgical effects had dissipated typically had a good post-operative course with few kidney infarcts. In addition, a target of 20% platelet activation would not require complete dissolution of pump induced platelet activation, but would be less than what was observed in the preclinical evaluation of the Heartmate II device, which despite being done in the bovine model is the only relevant preclinical study for comparison.

The platelet activation assays employed in this study have the potential to be valuable in comparing various designs, cannula configurations etc, which might not appear different in comparison studies evaluating only serum chemistry values. This phenomenon was observed in the bovine model where platelet activation assays similar to those employed in this chapter could detect differences the level of platelet activation in VADs with different surface coatings whereas the serum chemistry and hemorheology values could not detect meaningful differences in the surface coatings [62, 100].

The temporal course of platelet activation (**Figure 7-6**) in the "few/no infarct" group appears to be less than that observed in the uncomplicated cohort of studies using the commercially available cannula (**Figure 7-4**). This suggests the potential for more promising biocompatibility for the PediVAS using the custom cannula when compared to the commercially available cannula. However, since there were a moderate number of kidney infarcts and/or some deposition in the cannula more studies may be prudent to ensure the safe biocompatibility of the PediVAS in children with the custom cannulae set. Further investigation into how to prevent the elevated kidney infarcts seen in some of the studies might include ways to apply a biomimetic coating to the pump or the tubing or investigate ways to reduce the amount of tubing used in PediVAS implants to reduce the blood contacting surface area. In summary, the PediVAS was successfully implanted and met hemodynamic requirements with both cannulae sets. Platelet activation trended with complications and was elevated to a greater extent when there were more kidney infarcts and/or thrombotic deposition in the cannula demonstrating the sensitivity of the platelet activation assays.

7.4 CONCLUSIONS

The Levitronix PediVAS was successfully implanted in 20 animals and met its hemodynamic goals in successfully pumping ovine blood in the desired flow range of 0.5 – 1.5 LPM. Platelet activation assays generally trended with animal and pump complications as well as kidney infarct data. These assays also appeared to be more sensitive than traditional clinical markers of renal function with respect to thrombotic kidney damage. Platelet activation using the custom cannula holds promise in that a subset of these studies returned to baseline in a temporal course similar to VAD sham surgeries. Overall in the majority of studies platelet activation returned to baseline levels by 30 days which is an encouraging biocompatibility result; but more preclinical studies may help to enhance biocompatibility during clinical trials.

8.0 SUMMARY

8.1 OVERALL CONCLUSIONS

Bleeding and thromboembolism are major complications associated with VAD placement and hinder more widespread use of these devices. Pediatric VADs under development must have diminished levels of complications when implanted in children. The preclinical animal model for pediatric VADs is the juvenile ovine model. In this report a number of platelet activation assays were developed to quantify activated ovine platelets and function. These assays were able to demonstrate lower platelet activation results on biomimetic coated titanium surfaces versus uncoated titanium surfaces. Stress artifact observed during pre-operative blood collection was significantly reduced after placement of a jugular vein catheter. VAD surgical sham studies indicated that platelet activation from the implant surgery dissipated within approximately two weeks. During in vivo studies platelet activation assays were successfully used to assess pump performance. During in vivo studies, platelet activation consistently rose following pump or animal complications even when hemolysis results remained within normal limits. The level of platelet activation also was generally higher in studies where there were a number of kidney infarcts or thrombus deposition within the pump circuit when compared to studies that had few or no kidney infarcts. Even when there were numerous kidney infarcts along with elevated platelet activation, kidney serum chemistry values typically remained normal, suggesting that the

developed platelet activation might be more sensitive in assessing pump performance than the standard serum chemistry studies. In several of the PediaFlow and Levitronix studies platelet activation rose after surgery before returning to sustained baseline values after the surgical effect period had dissipated indicating that these pumps were causing minimal platelet activation. These results were further verified in vivo when low platelet activation was accompanied by an ability to respond to exogenous agonist stimulation indicating preserved platelet function. This low level of activation (before exogenous stimulation) may bode well for potential clinical use of the Levitronix PediVAS and PediaFlow VAD. In this study a number of ovine platelet activation assays were developed that demonstrated sensitivity in assessing surface coatings and pump performance. As a part of comprehensive design and evaluation of prospective blood contacting artificial organs the developed assays would be of significant value to improve biocompatibility of artificial organs.

8.2 FUTURE STUDIES

The results from this work have demonstrated that a number of ovine platelet activation assays have been developed. These assays proved to be very useful during in vitro and in vivo assessment of the pediatric VADs and suggest promising results for the potential clinical use of the PediaFlow and Levitronix PediVAS. There are a number of studies that could be done to build upon and enhance what has been learned from this report.

While the platelet activation assays used throughout this work proved to be of great value and utility, one drawback is that many of the results presented here evaluated the p-selectin

expression marker on ovine platelets. Multiple anti-p-selectin antibodies were used that had different clone numbers which suggested that each antibody bound to a different epitope on the p-selectin protein, providing some diversity within the assay. It must be mentioned that p-selectin is one of the most important cell surface markets on activated platelets and the one most extensively examined. However, this work would benefit from the development of other markers that could detect different activation markers on the ovine platelet surface. Cell surface markers such as CD63, released from dense granules of platelets upon activation, and the activation epitope on the platelet fibrinogen receptor could be investigated for ovine platelet activation assays. To our best knowledge antibodies that recognize or cross-react to these markers on ovine cells do not yet exist. However, some companies such as AbD Serotec offer services where an antibody can be created for a particular antigen and this service may be of benefit for these ovine studies. Enzyme-linked immunosorbent assays (ELISAs) to measure ovine platelet activation markers in serum would also enhance biocompatibility assessment in the ovine model. ELISAs depend on antibodies to recognize the epitope of interest and given the dearth of antibodies that bind to ovine antigens ELISA evaluation of ovine platelet activation is limited. Some meaningful markers to assess via ELISA would include platelet factor-4, β-thromboglobulin, thrombin anti-thrombin complexes, and prothrombin fragment F1.2. The services mentioned through AbD Serotec to raise antibodies against cell surface antigens might be a useful starting point in developing ELISAs to detect platelet activation markers in serum.

In this report pre-operative platelet activation was elevated in jugular venipuncture samples compared to samples taken from a venous catheter. The elevated platelet activation was attributed to stress, however it would be interesting to note if serum markers or hormones were

elevated after blood draw. A comparison study of serum cortisol and epinephrine levels following both types of blood collection could provide further insight into the cause of elevated platelet activation following jugular venipuncture and perhaps yield other ways to reduce artifact in pre-operative blood sampling.

In the surface coating studies the MPC surfaces were studied using $TiAl_6V_4$ square pieces in a hematology mixer whose flow regime did not mimic conditions seen in pediatric VADs. The next step would be to coat the blood contacting surfaces of a pediatric VAD and run simultaneous mock circulatory loops with and without the coating to assess it effectiveness. If the coating is successful in vitro (i.e., decreased platelet deposition and activation), in vivo studies with the pumps would be necessary to assess platelet activation on pumps with and without the blood contacting surface coatings, to further demonstrate its utility for potential clinical application in blood pumps. Combining superior VAD design (magnetically levitated and actuated impeller, a flow regime without vortices, and with no or minimal areas of recirculation and stagnation) with a superior surface coating strongly resistant to platelet deposition could significantly improve outcomes in VAD patients and ultimately enable more patients to benefit from this life saving technology.

Although the VAD community is increasingly moving to rotary blood pumps, there is much still to learn about the effects of these supraphysiologic shear environments on blood elements. Development of a blood shearing instrument that mimics a pediatric VAD similar to what was developed by Wu et al coupled with the assays developed in this report to determine tolerable shear stress and exposure times would greatly aid the understanding of flow effects on

blood cells [139]. More comprehensive in vitro studies performed with the PediaFlow and PediVAS examining the effects of different flow rates and even comparison studies of devices with and without contact bearings would further improve our understanding of blood biocompatibility. Results from such work could then be compared with CFD models and enable refinement of CFD models to better predict platelet behavior and perhaps improve the selection of the most biocompatible pump designs and improve the biocompatibility of the next generation of VADs.

A major objective of this work was to assess leukocyte activation following VAD implant. Assays to detect granulocyte and monocyte platelet aggregates appeared to increase following agonist stimulation but the isotype control antibody binding also increased suggesting that the developed GPA and MPA assays may not be meaningful. Also, when the leukocyte platelet aggregate assay was used, it did not appear to be very sensitive to the VAD implant. Further investigation should be performed in vitro to determine if this assay is viable as a measure of thrombosis/inflammation in the ovine model. A neutrophil oxidative burst assay may merit investigation to assess potential changes in granulocyte function after VAD implant, this type of assay does not require antibodies to particular activation markers on the granulocyte, which theoretically suggests that it may work in the ovine model. More encouraging in vitro results were obtained looking at markers for CD4 T-cell activation, while there was some inconsistency in the results and these markers did not reach statistical significance. Additional study is merited here, agonist incubation time for example could be varied (i.e. 24 hrs, for example) and more consistent results might be achievable. In vitro assessment of CD11b, also

thought to be expressed on activated T-cells may also be useful and may eventually become a meaningful in vivo T-cell activation assay.

A recent paper looked at cross reactivity of human antibodies to ovine antigens and one of the antibodies shown to cross-react to ovines (anti-human CD86, a B-cell activation marker) is of interest given the reports of B-cell hyperactivity in some VAD patients. There is increasing interest in the study of ruminant immunology, and over time it is expected that more markers will become available that recognize ovine markers of leukocyte activation. Activation-induced T-cell death as previously mentioned has been observed following VAD placement. In addition to its use as a platelet activation marker, Annexin V, also can be used to measure apoptosis, further assessment of Annexin V to measure leukocyte apoptosis assay may provide greater insight into pediatric VAD biocompatibility. In the **Appendix** lymphocyte activation following PediaFlow and Levitronix PediVAS placement is presented. The majority of these results show limited elevation of lymphocyte activation following implant. However, each of these studies incorporated prolonged use of antibiotics past the first week of surgery, which would be considered atypical for a VAD patient in the absence of infection. Evaluating lymphocyte activation during in vivo studies without prolonged prophylactic antibiotics may give a clearer understanding of the temporal response of lymphocyte activation following VAD placement. To better understand ovine immunology, measuring lymphocyte activation in ovines (not implanted with VADs) with known infection may provide the necessary insight into lymphocyte activation response and enable further interpretation of lymphocyte activation results when ovines are implanted with a pediatric VAD.

It comes as no surprise that there are very few ELISA kits available specifically for sheep. There are increasing numbers of antibodies that detect serum markers for inflammation that have become available from AbD Serotec and could be used as apart of a sandwich ELISA to quantify the inflammatory response following pediatric VAD implant, provided the appropriate positive controls can be developed to test their sensitivity. These anti-ovine antibodies for ELISA use include IL-1β, IL-6, and IL-8. Other antibodies that cross react to sheep include anti-TNF-α, TGF-β, and IL-12, IL-4, and IFN-γ.

As the PediaFlow and PediVAS devices move closer to clinical trial, continued in vivo biocompatibility assessment as discussed in this report is necessary to ensure that biocompatibility remains promising. In the case of the PediaFlow, a fourth generation device is under development that will require extensive biocompatibility characterization. In the most recent cohort of PediVAS studies numerous kidney infarcts were observed in half of their custom cannula studies, more work is necessary to see if these infarcts can be reduced. To further illustrate their biocompatibility some of these preclinical in vivo studies could be run without anticoagulation. Measurement of platelet life span would also be an intriguing study for subsequent in vivo experiments given the report by Snyder et al of decreased platelet lifespan following VAD implant [63]. There are other devices where the developed platelet activation assays could be used to assess biocompatibility. The PediVAS for example can be deployed as the pump head in an ECMO circuit and given its potential clinical use; the biocompatibility of this circuit must be evaluated before it would be approved by the FDA. Other devices that could potentially benefit from the preclinical biocompatibility assessment using assays developed in

this report include the Levitronix integrated pump oxygenator and the pediatric Jarvik pump, as well as novel heart valves under development.

The ultimate arbiter for success of the PediVAS and PediaFlow pump will be how these devices perform clinically. In the animal model, both pumps showed promise, however it is unclear how the sheep platelet response to these devices will compare to the human platelet response. In vitro mock circulatory loops with human and ovine blood set up for each pump would provide meaningful comparison studies and could provide insight into the comparative response of each species. Further information would be gained in clinical studies where the same markers of activation used in the preclinical ovine model are applied during clinical trials. This can be done in that a number of the platelet activation assays developed in this report actually target human antigens. Attaining a greater understanding of how sheep platelet response compares to human platelet response can strengthen our animal assessment methods to ensure that the most biocompatible devices enter the clinic.

APPENDIX A

IN VITRO ASSESSMENT OF ANTI-HUMAN PLATELET ACTIVATION ANTIBODIES

Table 8-1: Anti-human platelet activation antibody binding to ovine platelets

Anti-human Platelet Monoclonal Antibodies; N ≥ 2

Manufacturer:	Serotec	Beckmann Coulter	Santa Cruz
Clone #:	MEM-259	CLBGran/12	Polyclonal
Antigen Target:	Human CD63	Human CD63	Human CD62P
Quiescent	4.3 ± 0.5	3.8 ± 2.0	11.9 ± 5.5
20 μM ADP	6.4 ± 2.7	5.5 ± 2.2	12.4 ± 1.1
10 μM PAF	9.9 ± 4.0	6.2 ± 1.8	12.0 ± 2.2

APPENDIX B

IN VIVO SHAM STUDY

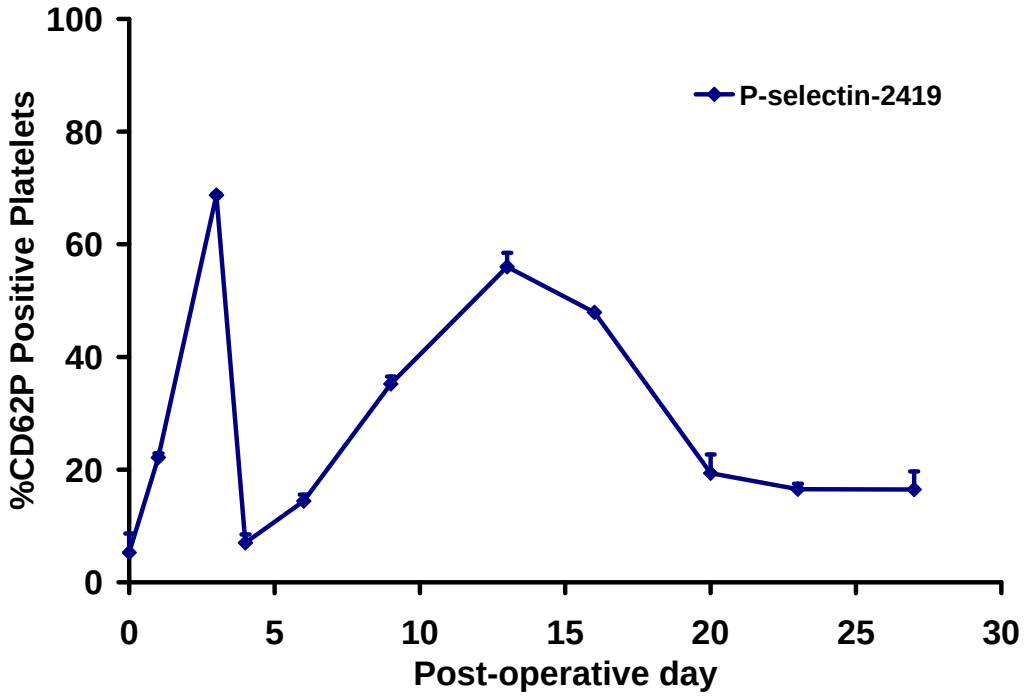

Figure 8-1 Platelet Activation following the 6th sham study

In this study platelet activation was higher than what was observed in the sham surgery cohort of studies, seen in **Figure 5-5**. Platelet activation did not return to baseline. It is worth noting that this animal was not on heparin as the other sham animals were because of suspected

bleeding. A thrombus was observed in the right atrium, which grew around the jugular venous line, at necropsy. Some of the TEG parameters were also elevated early on in the post-operative period of this study.

APPENDIX C

IN VIVO BIOMEDICUS STUDIES

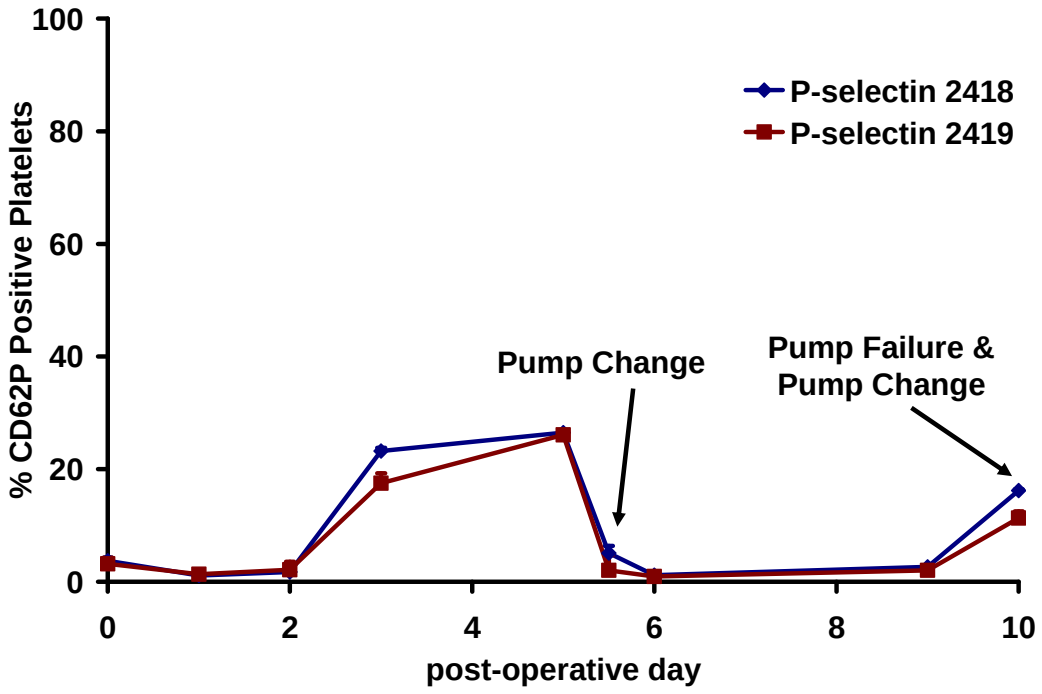

Figure 8-2: Platelet Activation following initial Biomedicus implant

On day 5, a pump change was executed due to very high hemolysis rates. As soon as the initial pump head was changed, platelet activation decreased back to baseline. There was substantial bearing thrombus observed in the initial pump head. A moderate rise in activation was observed following pump failure on day 10.

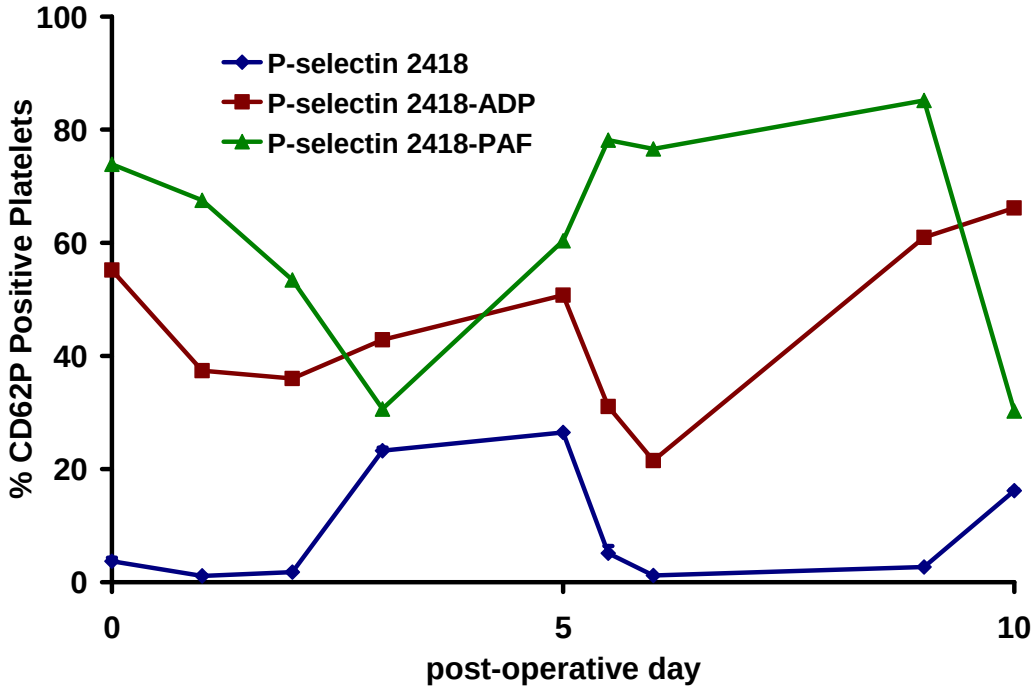

Figure 8-3: Platelet Activation following stimulation after initial Biomedicus implant

Although platelet activation was not as high as in other studies in the first week after surgery, poor response to stimulation especially evident in the inconsistent PAF results may have been the result of platelet damage caused by the pump.

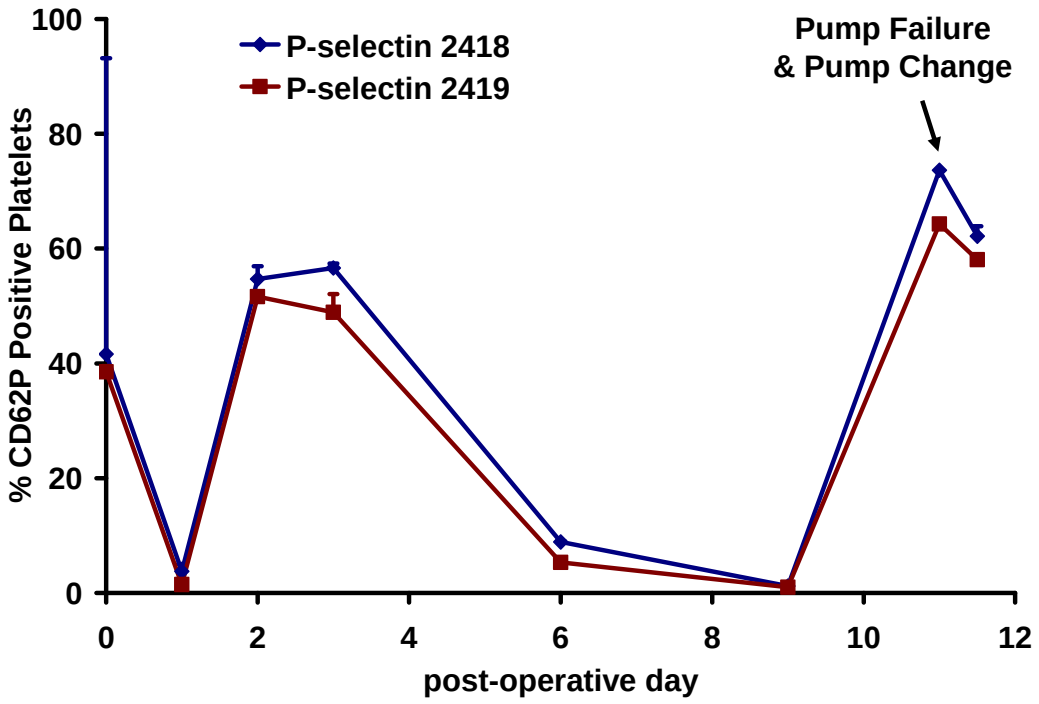

Figure 8-4: Platelet Activation following 2nd Biomedicus implant

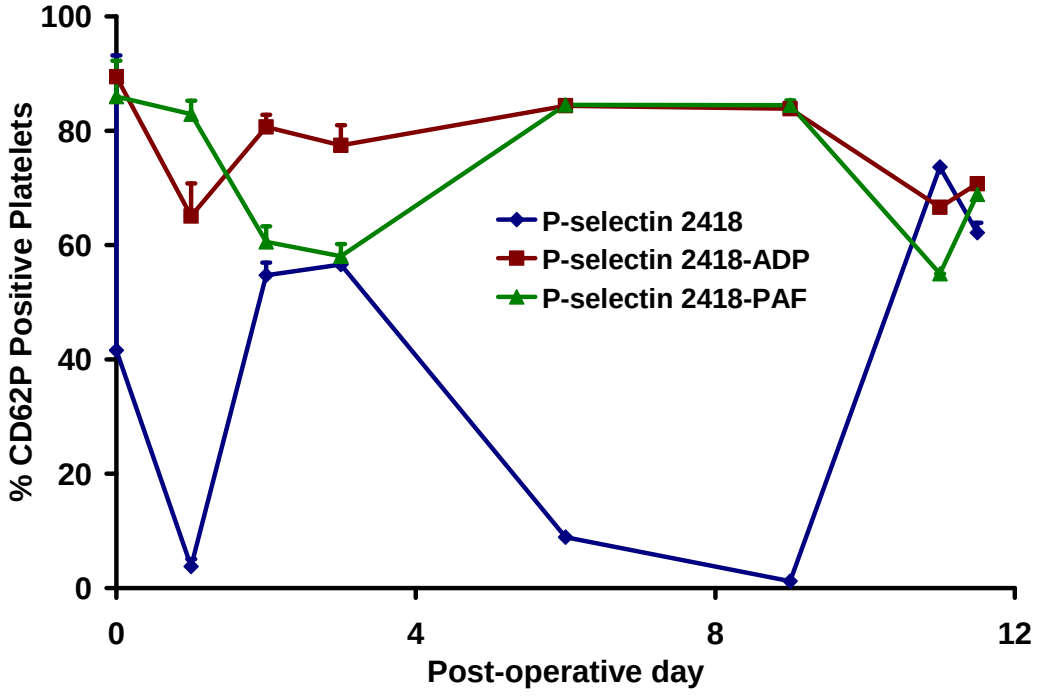

Figure 8-5: Platelet Activation following stimulation after 2nd Biomedicus implant

Platelet activation rose on the second post-operative day, returning to baseline on day 6. In this study platelet response to stimulus was as expected. On day 11, there was cavitation and a pump failure which likely led to a large spike in platelet activation on the final day of the study.

APPENDIX D

TODDLER VAD (TVAD) PLATELET ACTIVATION STUDIES

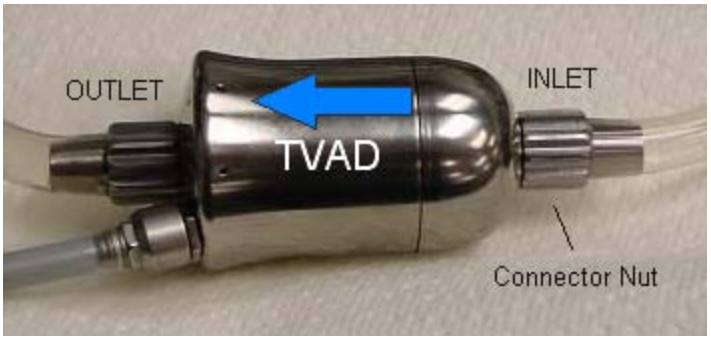

Figure 8-6: Toddler VAD, courtesy of Dr. James Antaki

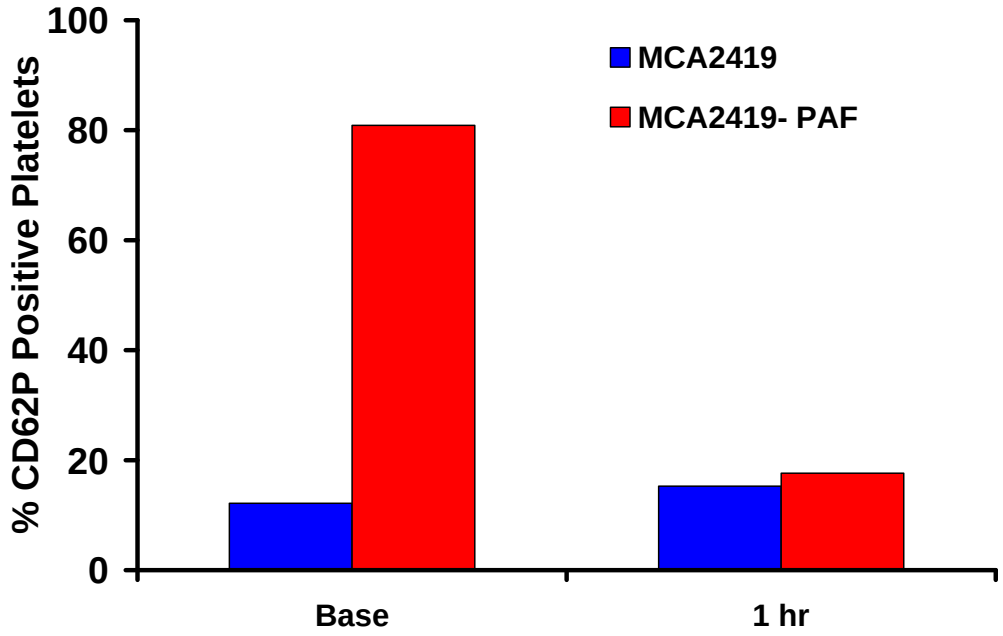

Figure 8-7: Platelet Activation after 1 hr Toddler VAD in vitro test

In this study platelet activation was low and could respond to a high degree to PAF stimulation. After a 1 hr pump run however, platelets lost all of their ability to become activated and further to respond to PAF stimulation.

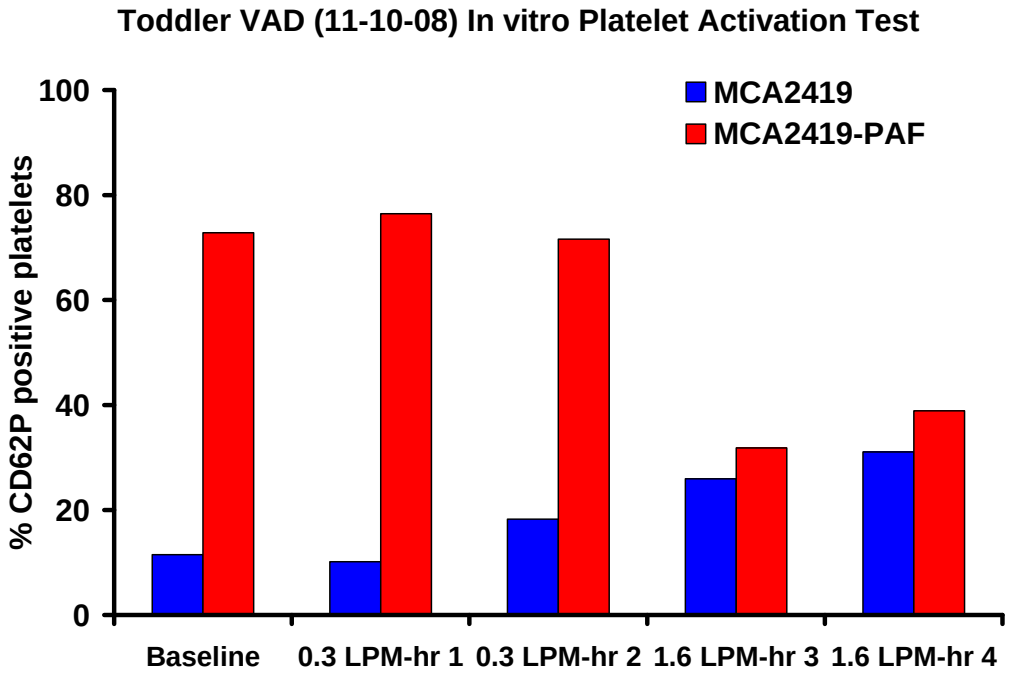

Figure 8-8: Platelet Activation following 4 hr TVAD in vitro test

At the low flow rates platelet activation was low and able to respond to PAF stimulation, a promising sign. At the higher flow rates however, there was more activation with a loss in ability to respond to PAF stimulation. The TVAD has an updated axial thrust bearing compared to the TVAD studied in the previous figure: **Figure 8-7.** Since the TVAD was able to run for 4 hours and 2 hrs at the higher speed and showed an ability to become activated and respond marginally to PAF stimulation it can be considered an improvement from the previous bearing.

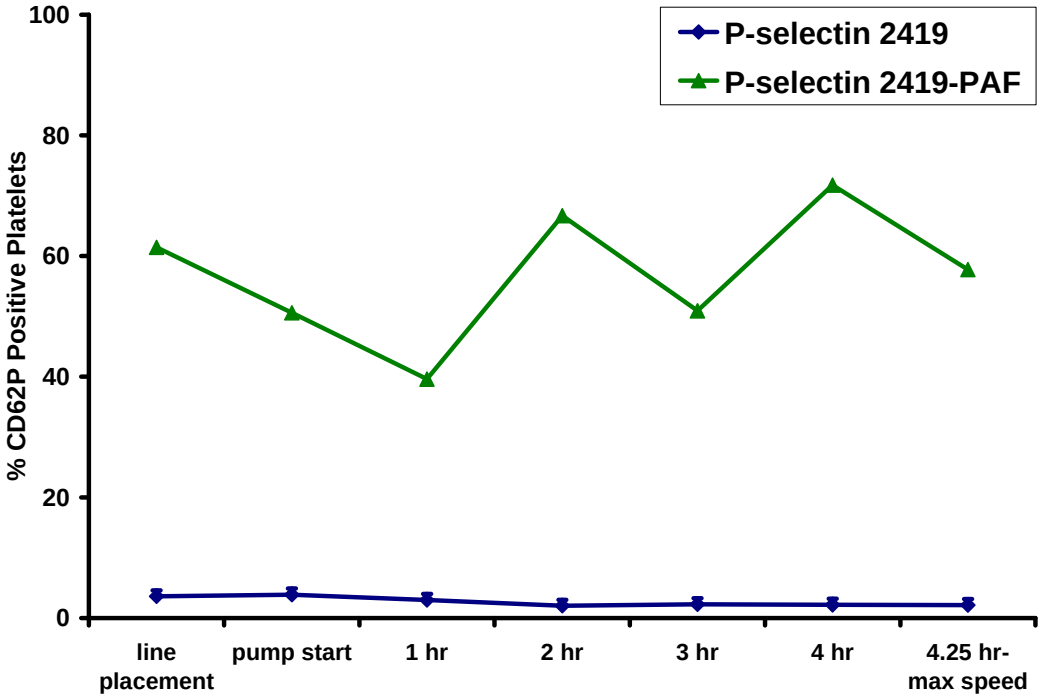

Figure 8-9: Platelet Activation following acute implant of the Toddler VAD

The low platelet activation observed in this study along with the demonstrated ability of platelets to respond to agonists after contact with the TVAD in a 4 hr acute study was promising. In this study the TVAD again possessed an updated axial bearing when compared to the TVAD bearing used in: **Figure 8-7** and **Figure 8-8.** The results in this study build upon the improvements in the previous bearing from **Figure 8-8.**

APPENDIX E

IN VIVO LYMPHOCYTE ACTIVATION

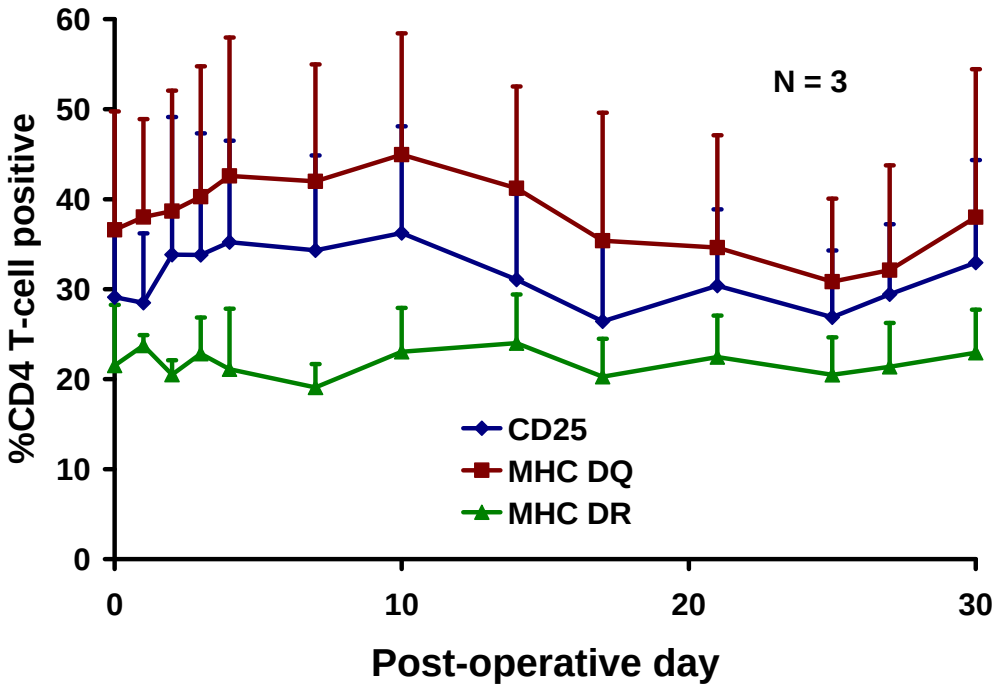

Figure 8-10: CD4 T-cell activation following sham surgery

There was a modest elevation in CD4 T-cells expressing CD25 and MHC DQ in the first week following surgery. MHC DR expression on T-cells remained stable throughout the temporal course. It was surprising that there was only a minimal rise in activation following sham surgery, whereas there was a definite rise in platelet activation (**Figure 5-5**). Also of note

were the relatively high levels of baseline lymphocyte activation. As illustrated in **Figure 5-3** the placement of a jugular venous catheter significantly reduced baseline platelet activation. The line placement did not decrease lymphocyte activation. One potential reason for elevated activation is that the animals may have been fighting a low grade infection.

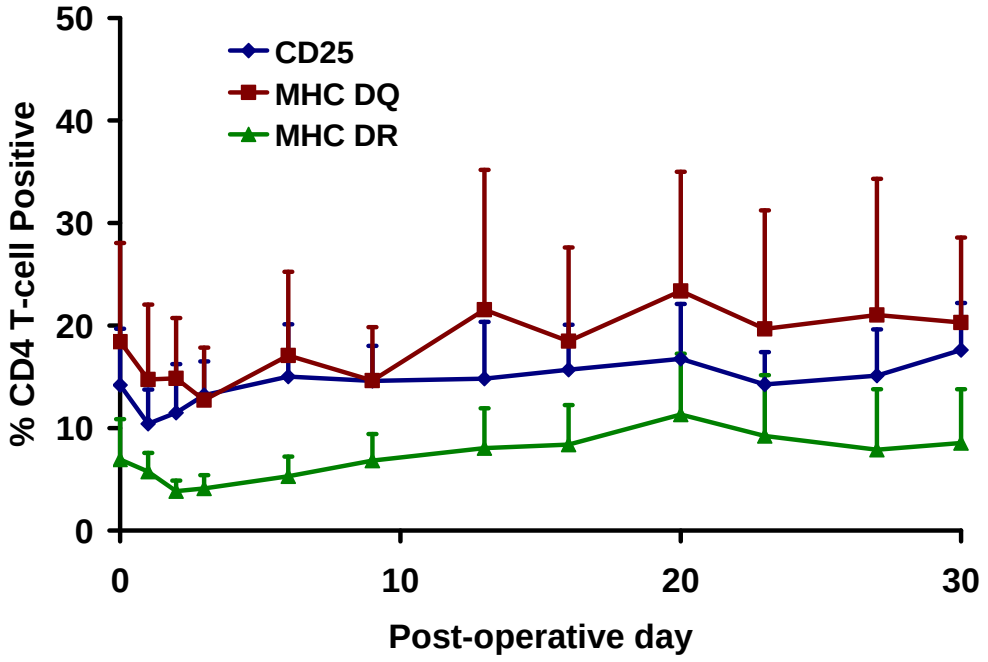

Figure 8-11: CD4 T-cell Activation after Levitronix implant; N = 6

After Levitronix PediVAS implant lymphocyte activation was stable for the thirty day period. One animal from this cohort was suspected to have an infection as evidenced by a neutrophil/lymphocyte switch late in the implant period. The lack of an elevation in T-cell activation was somewhat surprising. It is thought that lymphocyte activation might be a precursor to infection in these studies. Given that the Levitronix has two open wound sites for the inflow and outflow tubing, these animals might be expected to have a higher incidence of infection. The Levitronix implants only lasted for thirty days and if these studies continued for longer time periods, it is possible that some of these animals may have become more prone to

infection and potential increases in lymphocyte activation. In adults with VADs the incidence of infection continues to rise with time. Also of note was that each implanted animal received prophylactic antibiotics for thirty days, further decreasing the potential for the VAD implanted animals to contract an infection. Given the increased shear observed in the Levitronix and PediaFlow blood pumps it was thought that more lymphocyte activation would be observed, as was observed with the platelets. Minimal increases in lymphocyte activation have to be considered to constitute a positive biocompatibility result.

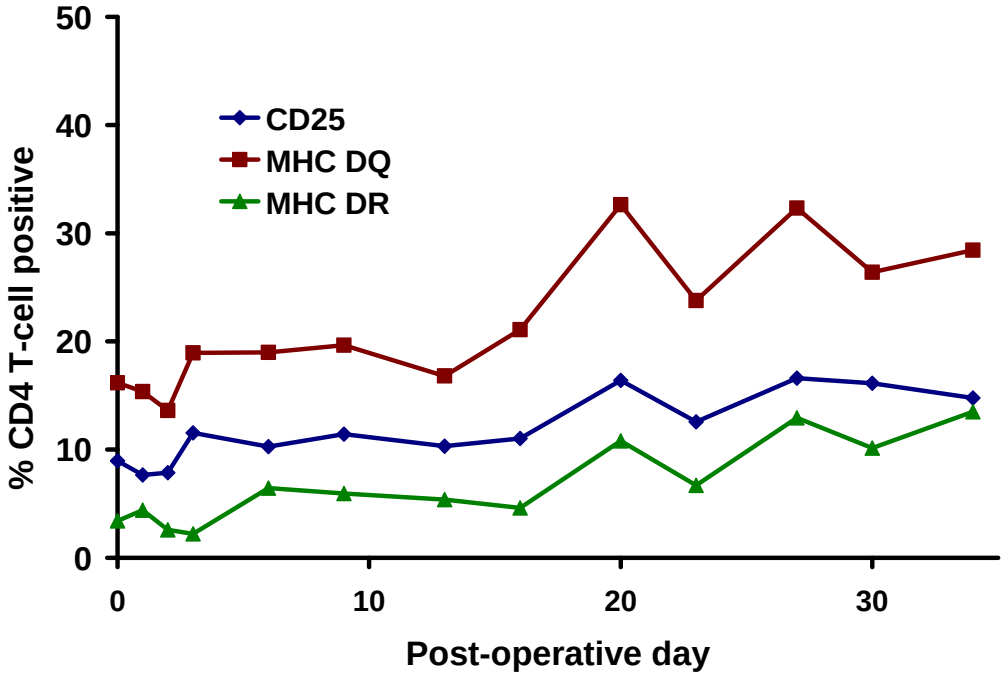

Figure 8-12: CD4 T-cell activation following Levitronix implant in animal with suscepected infection

In this singular study there was a trend towards increased lymphocyte activation and there was at least suspicion of a respiratory infection evidenced by the increased carbon dioxide and decreasing oxygen saturation and a neutrophil/lymphocyte switch. As mentioned above infection was not common in the Levitronix implants, however it is certainly possible that

lymphocyte activation might increase before an infection is detected, which would be similar to the observation of increased platelet activation and more numerous kidney infarcts at necropsy discussed in **Section 7.3.3.**

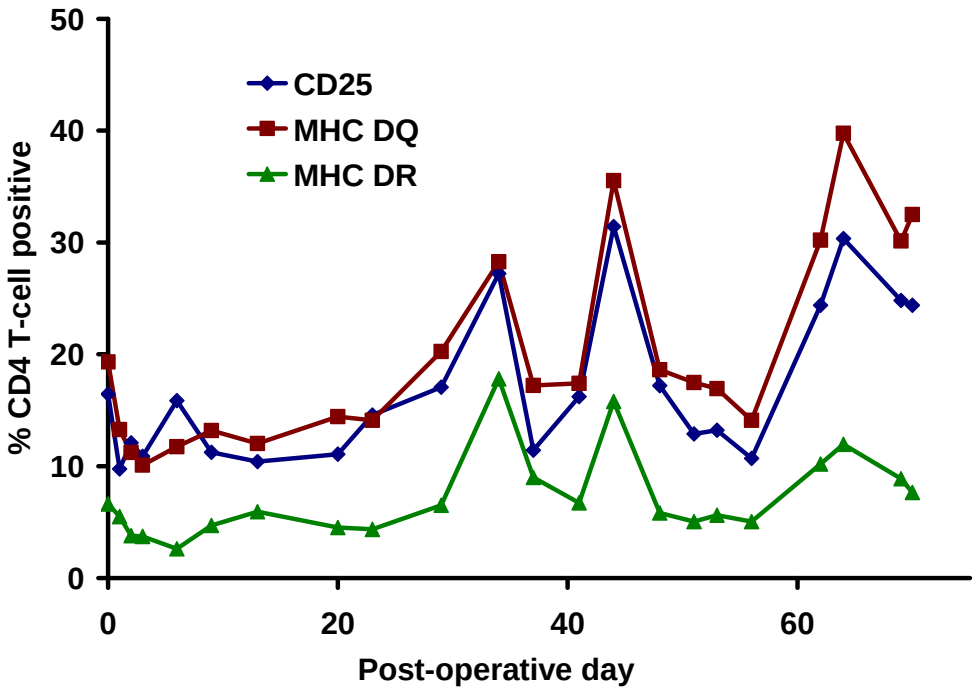

Figure 8-13: CD4 T-cell activation in seventy day PF2.2 implant

In this study a modest increasing trend is observed after the initial three weeks of implant. A fecal culture was positive for a nematode in this animal.

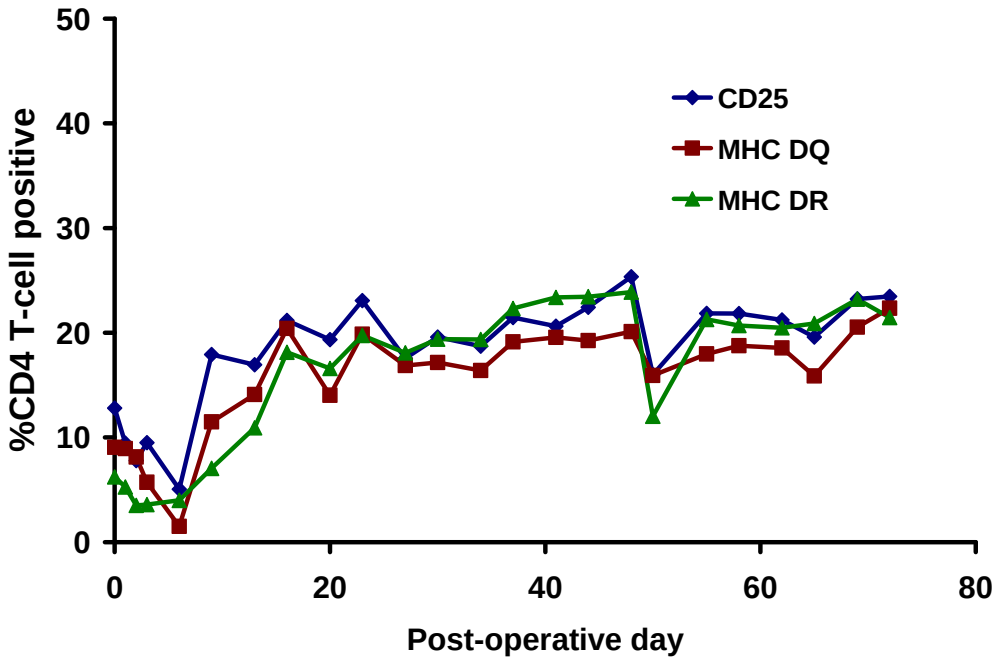

Figure 8-14: CD4 T-cell activation following PF3.1 implant

There was no evidence of ongoing infection in this study but a clear trend of increasing lymphocyte activation was noted.

APPENDIX F

TOTAL AVERAGE OF IN VIVO PLATELET ACTIVATION

This grand average was computed because it might be useful to use as a comparison for platelet activation for future in vivo studies. This average contains platelet activation values from all of the PediaFlow, Levitronix, Biomedicus, and sham studies where p-selectin was studied. As such it contains both data from studies without complications as well as data with complications. Since this average encompasses studies with and without complications it might represent a temporal course of platelet activation such that, when a study has platelet activation above this 'total average' it would be expected to have complications or numerous kidney infarcts and when a study has platelet activation below this "total average", the study would be expected to have no or minimal complications and few to no kidney infarcts. The data is further broken up into Levitronix and PediaFlow cohorts.

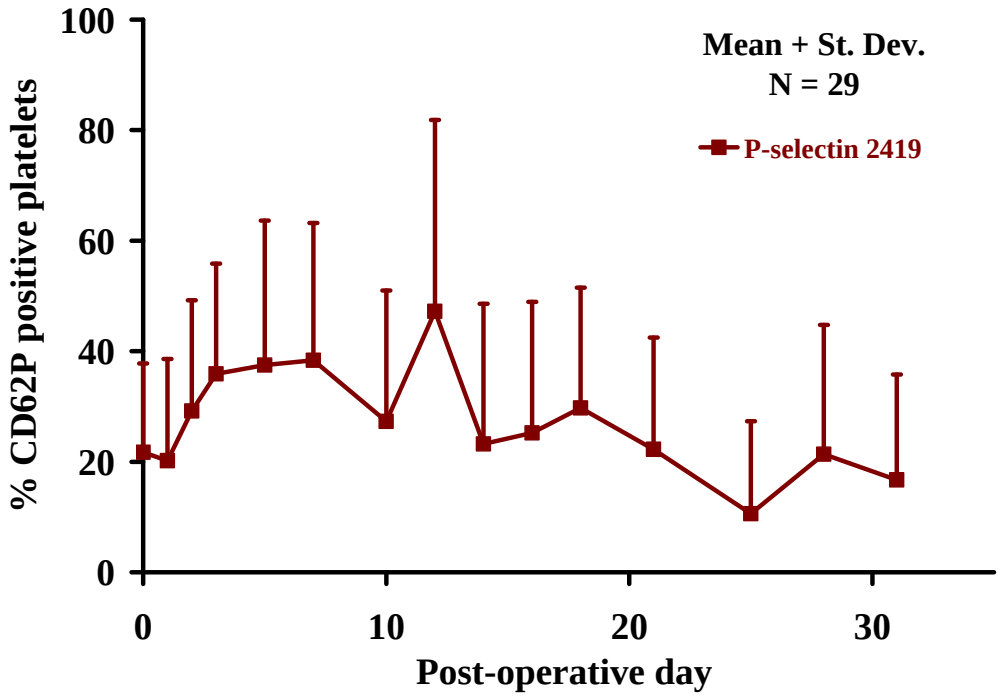

Figure 8-15: Combined platelet activation for all in vivo studies for first 30 days, N = 29

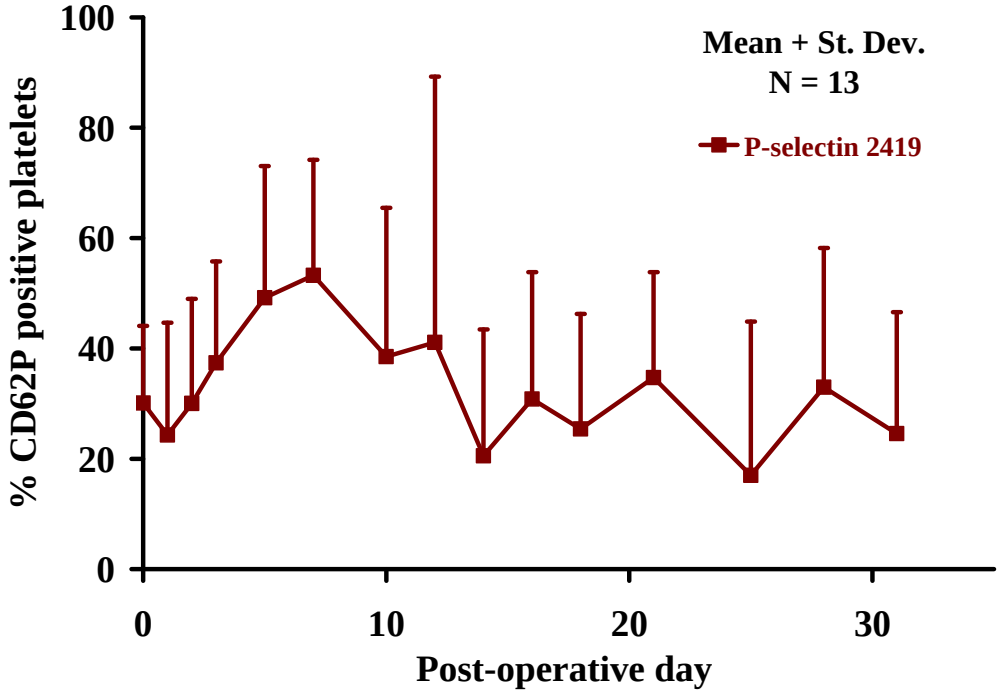

Figure 8-16: Combined platelet activation for all Levitronix data, N = 13

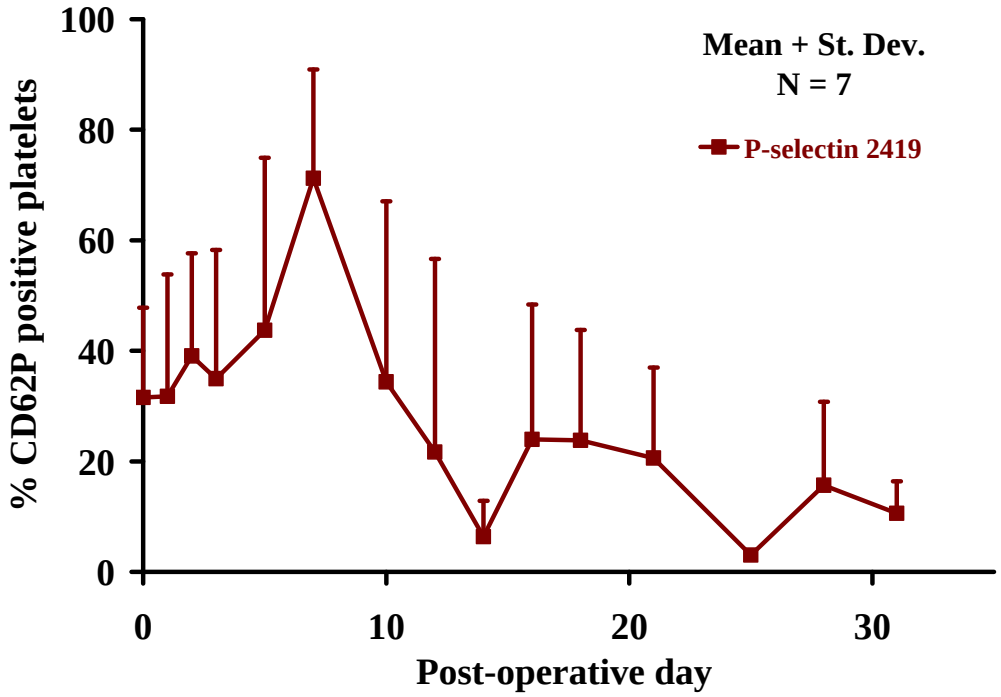

Figure 8-17: Combined platelet activation for Levitronix implant with uncomplicated post-operative courses

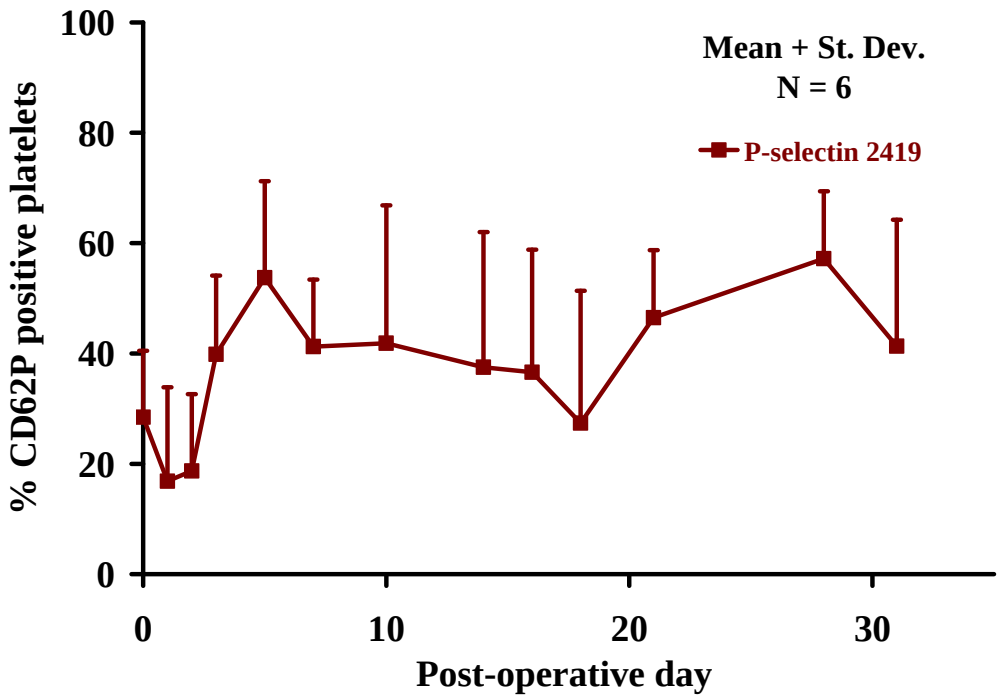

Figure 8-18: Combined platelet activation for Levitronix implants with complicated post-operative courses or in studies that had numerous kidney infarcts or thrombotic deposition in the cannula.

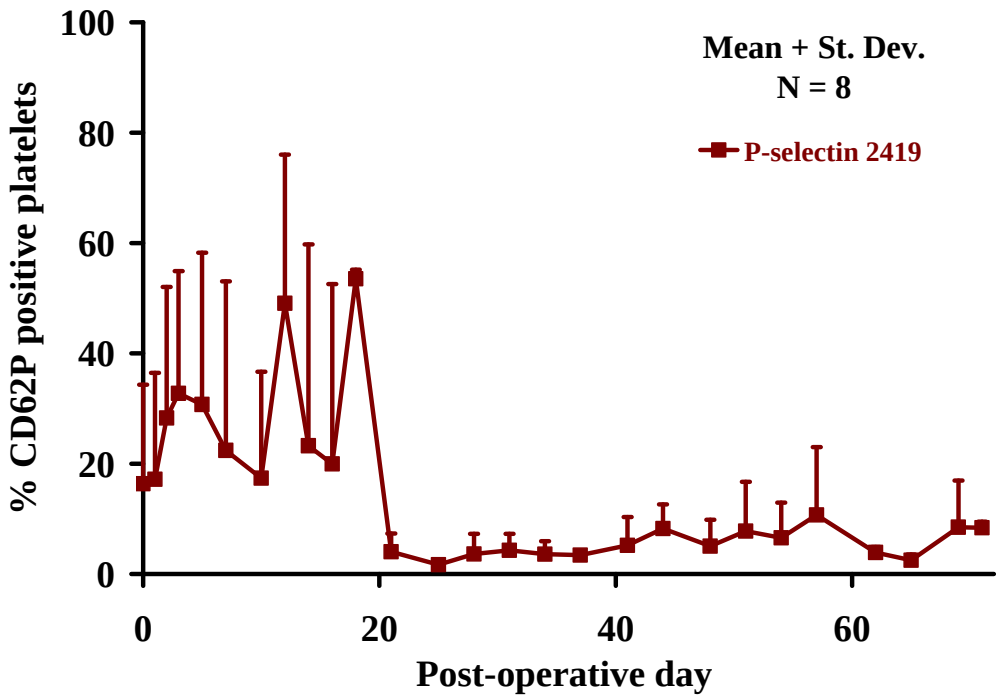

Figure 8-19: Combined platelet activation for all PediaFlow implants, N = 8

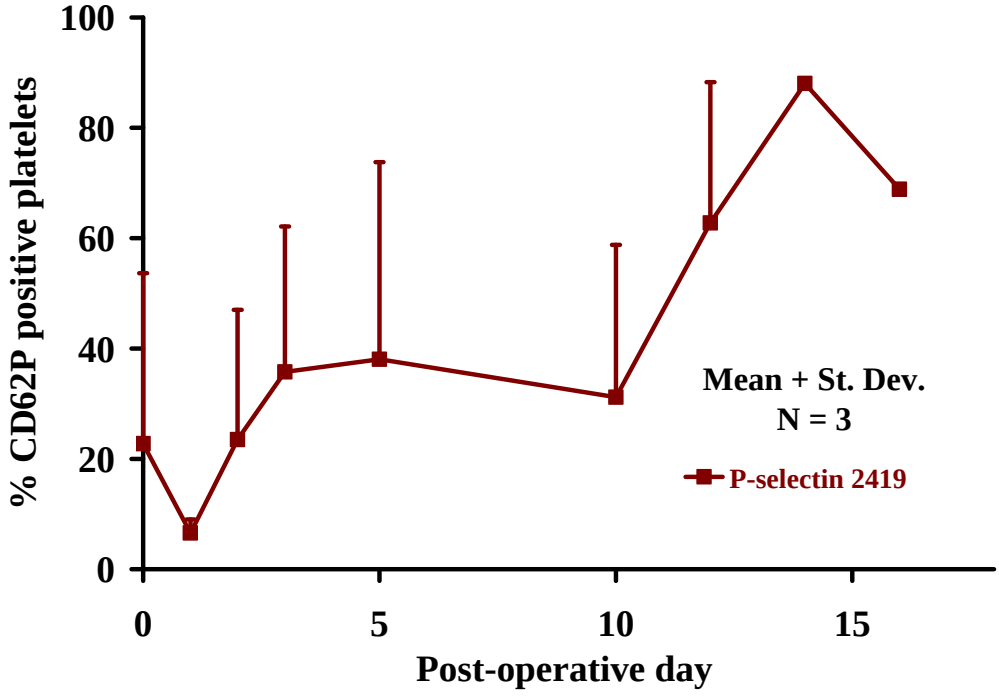

Figure 8-20: Combined platelet activation for PF1 implants, N = 3

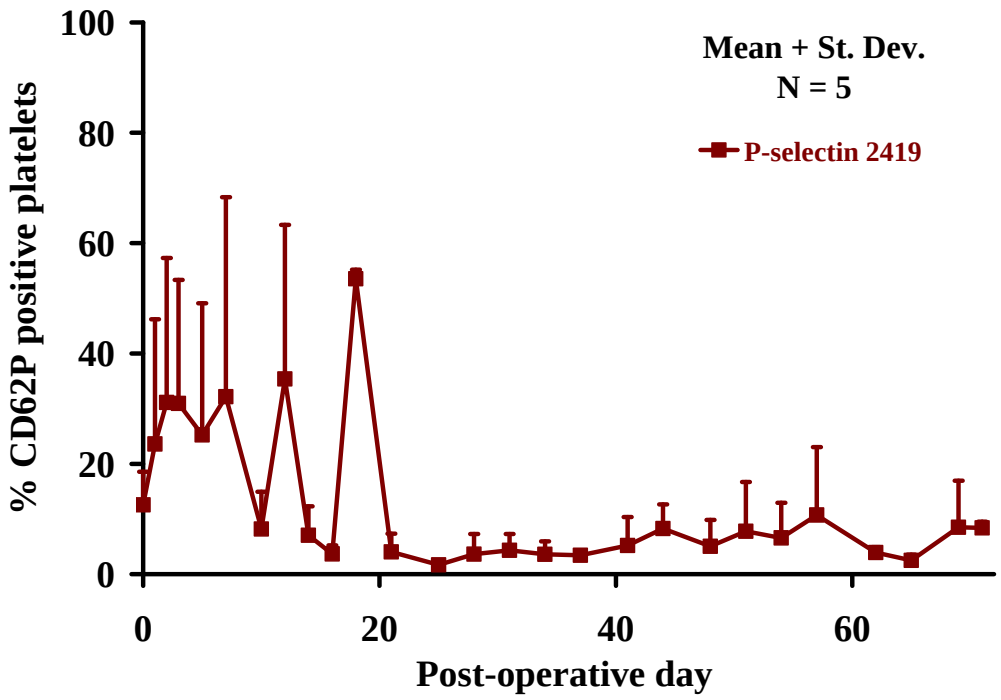

Figure 8-21: Combined platelet activation for PF2 and PF3 implants, N = 5

APPENDIX G

IN VIVO THROMBOELASTOGRAPH VALUES

In vivo TEG values (maximum amplitude and clot firmness (G) are plotted to determine if there were any trends in these values in Levitronix studies with and without post-operative complications.

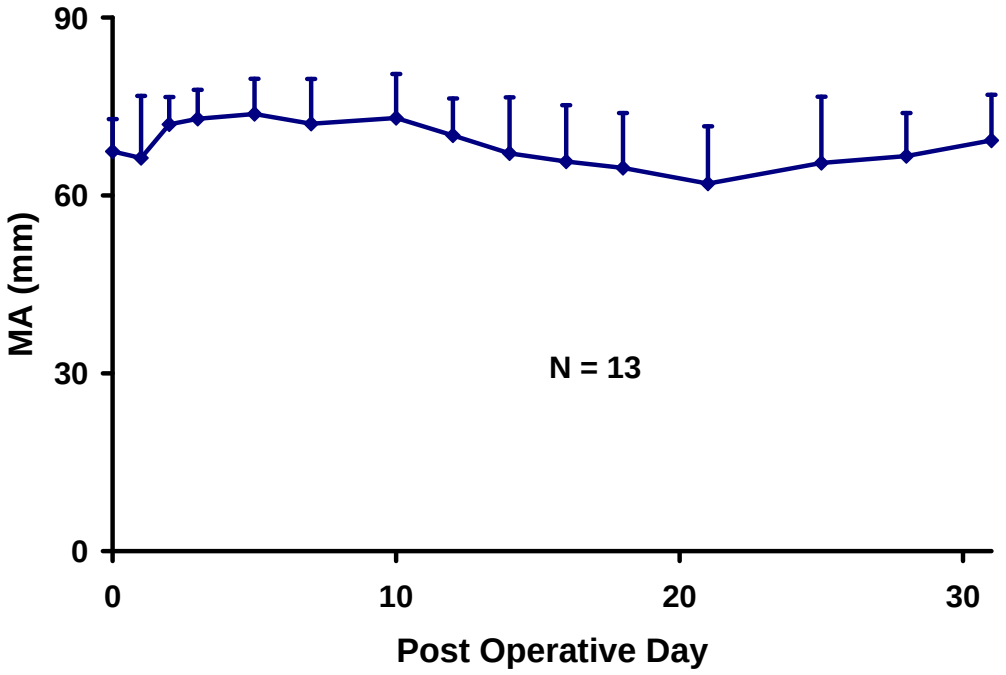

Figure 8-22: TEG MA values from the Levitronix cohort of in vivo studies

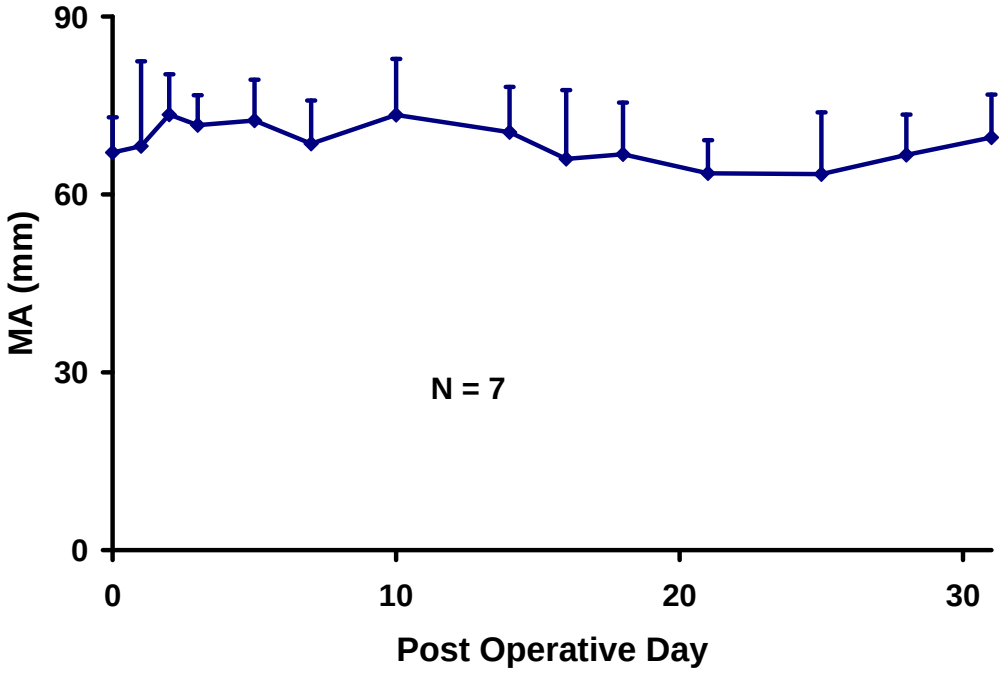

Figure 8-23: TEG MA values for Levitronix implants with uncomplicated post-operative courses.

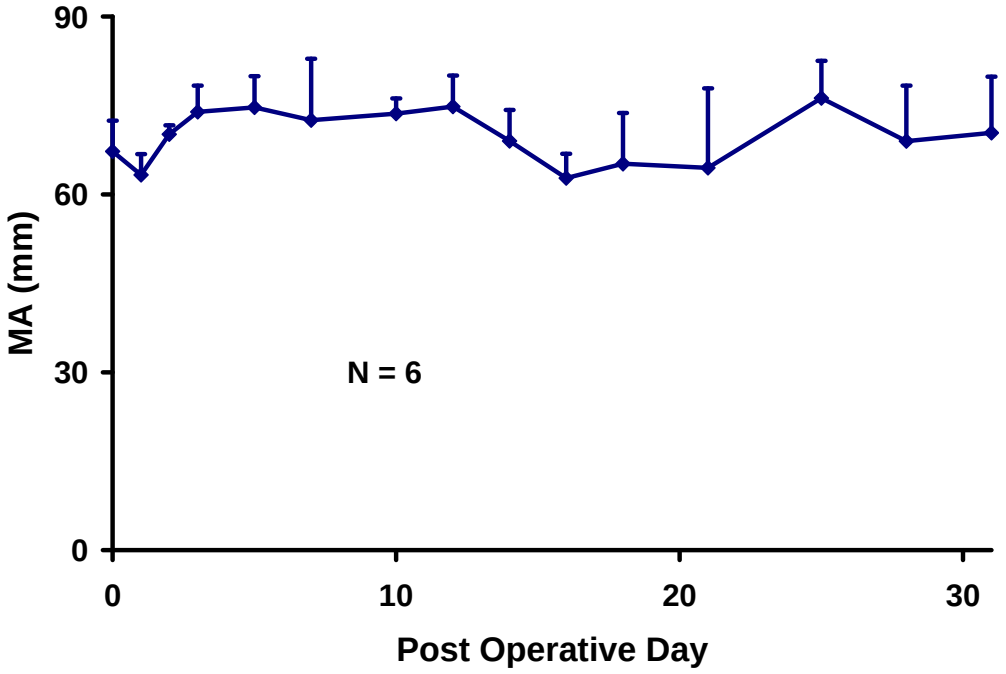

Figure 8-24: TEG MA values for Levitronix implants with complicated post-operative courses or in studies that had numerous kidney infarcts or thrombotic deposition in the cannula.

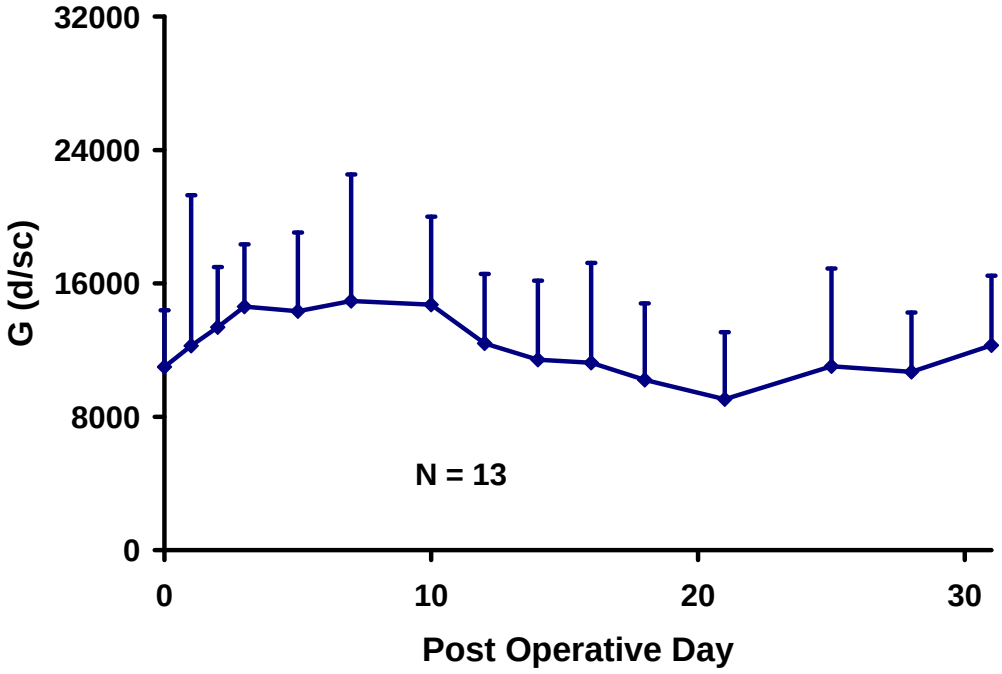

Figure 8-25: TEG G values from the Levitronix cohort of in vivo studies

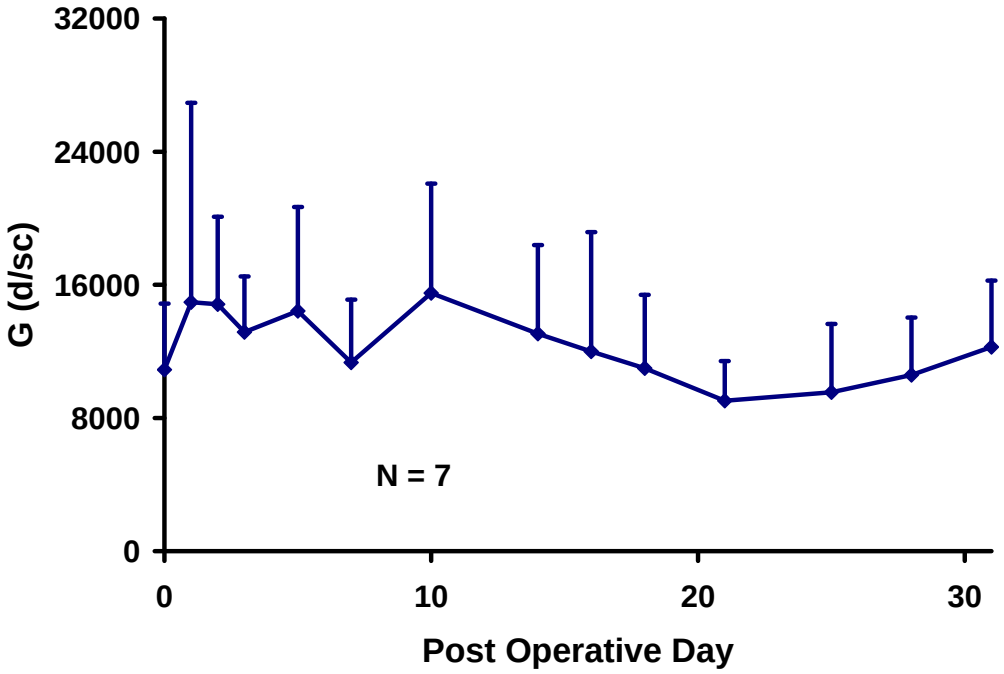

Figure 8-26: TEG G values for Levitronix implants with uncomplicated post-operative courses.

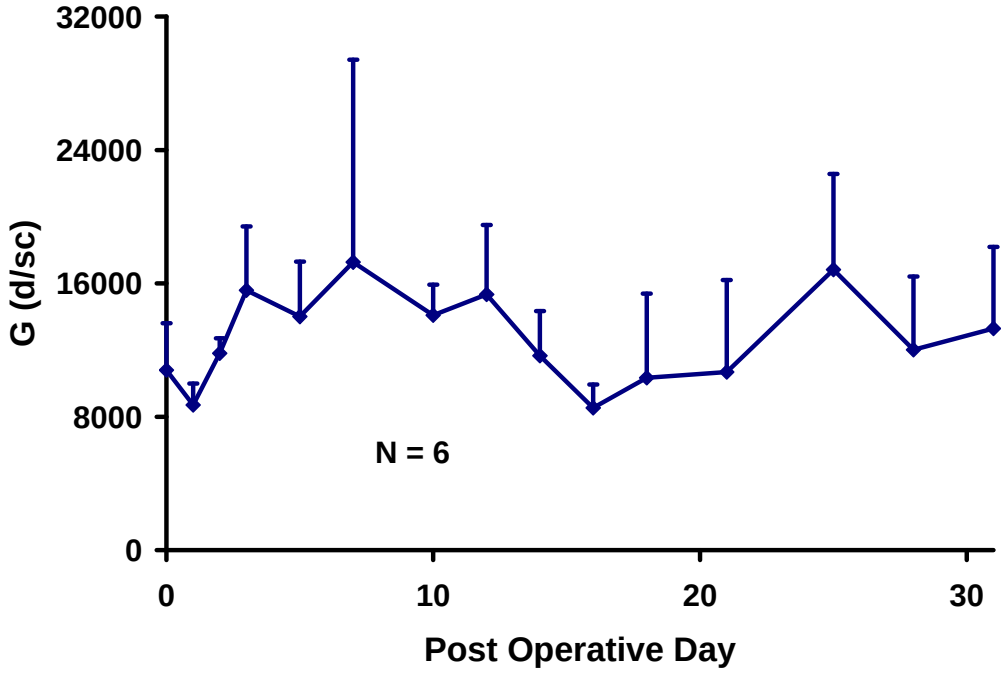

Figure 8-27: TEG G values for Levitronix implants with complicated post-operative courses or in studies that had numerous kidney infarcts or thrombotic deposition in the cannula.

Despite noticeable trends in the level of platelet activation (compare **Figure 8-17** and **Figure 8-18**) in studies with uncomplicated post-operative courses versus those studies that had complications or had numerous infarcts at necropsy, MA and G do not exhibit such trends. The lack of a trend for TEG values suggest that the developed flow cytometric platelet activation assays are more sensitive in assessing in vivo VAD biocompatibility.

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