THE PREOPERATIVE IMMUNE RESPONSE AS A PREDICTOR OF HEPARIN-INDUCED THROMBOCYTOPENIA AFTER CARDIOPULMONARY BYPASS SURGERY

UTILIZING THE PREOPERATIVE PF4-DEPENDENT IMMUNE RESPONSE TO PREDICT ANTI-PF4/HEPARIN ANTIBODY PRODUCTION IN A COHORT OF PATIENTS UNDERGOING CARDIOPULMONARY BYPASS SURGERY

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TITLE: Utilizing the preoperative PF4-dependent immune response to predict anti-PF4/heparin antibody production in a cohort of patients undergoing cardiopulmonary bypass surgery.

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ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) is an iatrogenic immune-mediated prothrombotic disorder that is a direct consequence of heparin therapy. In HIT, antibodies are generated against complexes of platelet factor-4 (PF4) and heparin. Immunoglobulin G (IgG) antibodies bind to PF4/heparin complexes and cause Fc-receptor-mediated activation of platelets and monocytes. PF4 binds endogenous heparin-like polyanions to reveal cross-reactive epitopes that can also bind anti-PF4/heparin antibodies. Based on this observation, researchers have suggested that exposure to PF4/polyanion complexes can sensitize immune cells to become activated to produce HIT antibodies following iatrogenic heparin exposure. *Research objective:* The objective of this study is to determine whether the preoperative PF4-dependent immune response is associated with postoperative anti-PF4/heparin antibody production in a cohort of patients undergoing cardiopulmonary bypass surgery. *Materials and methods:* To assess the preoperative immune response to PF4, we utilized two assays: (1) a ^{3}H-thymidine uptake assay to measure peripheral blood mononuclear cell (PBMC) proliferation in response to *in vitro* stimulation with PF4 and (2) a PBMC ELISPOT assay to measure the preoperative frequency of PF4-specific antibody-secreting cells. Proliferation was quantified as a stimulation index (SI). We then utilized a PF4/heparin-dependent enzyme immunoassay to measure the *in vivo* levels of anti-PF4/heparin antibodies produced by these patients in the postoperative period. *Results:* Our findings suggest that preoperative PF4-dependent proliferation is not associated with postoperative polyspecific anti-PF4/heparin antibody production [Spearman's ρ (95% CI) = –0.02 (–0.32, 0.28), $P = 0.91$]. PF4-dependent proliferation had a weak negative association with postoperative anti-PF4/heparin IgG antibody production [Spearman's ρ (95% CI) = –0.31 (–0.56, –0.02), $P = 0.04$], but was not associated with postoperative IgM or IgA anti-PF4/heparin antibody production [IgM:

Spearman's ρ (95% CI) = –0.04 (–0.33, 0.26), *P* = 0.78; IgA: Spearman's ρ (95% CI) = –0.05 (–0.34, 0.25), *P* = 0.73]. Qualitative analysis demonstrated that two patients who had the strongest preoperative PF4-dependent proliferation responses produced the highest postoperative levels of anti-PF4/heparin IgM antibodies, but this relationship was not observed with postoperative anti-PF4/heparin IgG antibodies. Moreover, the preoperative frequency of PF4-specific antibody-secreting cells (ASCs) was also not associated with postoperative levels of anti-PF4/heparin IgM or IgA antibodies [IgM: Spearman's ρ (95% CI) = 0.30 (–0.79, 0.93), *P* = 0.683; IgG: Spearman's ρ (95% CI) = –0.21 (–0.92, 0.83), *P* = 0.600]; however, this was only completed on five patients and so the sample size should be increased before any meaningful conclusions can be drawn. We also demonstrated that PF4-dependent proliferation increases 5–6 days following cardiopulmonary bypass surgery [geometric mean (GM) postoperative PF4 alone proliferation (in SI) vs. GM preoperative PF4 alone proliferation (in SI) ± SEM: 23.7 ± 1.3 vs. 6.9 ± 1.5, *P* = 0.009]. *Conclusions:* Based on our findings, we conclude that preoperative PF4-dependent proliferation is unable to predict postoperative anti-PF4/heparin antibody production in this cohort of cardiopulmonary bypass patients. Due to the small sample size, we are unable to make conclusive statements regarding the relationship between preoperative PF4-specific ASC frequency and postoperative anti-PF4/heparin antibody production, but our findings would suggest that an association does not exist between these two variables in this patient cohort. Cardiopulmonary bypass surgery, however, may mobilize the postoperative immune cell repertoire to become activated against the self-protein PF4 and may therefore contribute to the postoperative HIT immune response.

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LIST OF ABBREVIATIONS

ACD	Acid citrate dextrose
ASC	Antibody-secreting cell
BCA	Bicinchoninic acid assay
BCIP	5-bromo-4-chloro-3'-indoylphosphate
BSA	Bovine serum albumin
CPB	Cardiopulmonary bypass
CD	Cluster of differentiation
CI	Confidence Interval
CPM	Counts per minute
EdU	5-ethynyl-2´-deoxyuridine
EIA	Enzyme immunoassay
ELISPOT	Enzyme-linked Immunospot Assay
EU	Endotoxin units
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GM	Geometric mean
HPLC	High-performance liquid chromatography
HiREB	Hamilton Integrated Research Ethics Board
LAL	Limulus Amebocyte Lysate
LMWH	Low-molecular weight heparin
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex

OD_{40}	Optical density
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
pNPP	p-nitrophenylphosphate
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PHR	Protein-to-heparin ratio
R848	Resiquimod
SDS	Sodium dodecyl-sulfate
SI	Stimulation index
SD	Standard deviation
SEM	Standard error of the mean
UFH	Unfractionated heparin
UV	Ultraviolet

DECLARATION OF ACADEMIC ACHIEVEMENT

This study is the first known usage of clinical populations to investigate the effect of the immune response prior to heparin exposure on susceptibility towards developing heparin-induced thrombocytopenia (HIT). To address this research objective, we were required to consent cardiopulmonary bypass surgery patients prior to surgery for a preoperative and postoperative blood draw. Patient consenting and sample collection were completed by Phillip Staibano with the help of nurses at both McMaster University Medical Centre and the Hamilton General Hospital. In order to study the preoperative immune response, we employed two immunological assays: a PF4-dependent ^{3}H-thymidine uptake assay and a PF4-specific ELISPOT assay. For the patients recruited into this study, both assays were completed by Phillip Staibano. Rumi Clare and Ishac Nazi optimized both immune assays and manuscripts that address results from both assays are currently being drafted. Due to the heterogeneous nature of the clinical population utilized in this study, we also performed a retrospective chart review to characterize the demographic characteristics and gather more information about the medical histories of each of patients recruited into the study. Phillip Staibano submitted the HiREB study protocol for this objective and completed the chart review for each patient recruited into this study. To analyze postoperative anti-PF4/heparin antibody production, we utilized a PF4/heparin-dependent enzyme immunoassay, which was developed by the McMaster Platelet Immunology Laboratory and is currently utilized for diagnostic HIT testing. Although Phillip Staibano was trained on how to perform these assays, for this study, the majority of these assays were performed by Jane Moore and Sheila Baker of the McMaster Platelet Immunology Laboratory. All statistical analyses were completed by Phillip Staibano. The thesis was written and edited by Phillip Staibano.

Based on the results collected, we conclude that preoperative PF4-dependent proliferation is not associated with postoperative anti-PF4/heparin antibody production. Preliminary results also suggest that preoperative PF4-specific ASC frequency cannot predict postoperative anti-PF4/heparin antibody production within this cohort of cardiopulmonary bypass patients. These results suggest that neither of these assays can predict postoperative anti-PF4/heparin antibody production, but future studies should look into other preoperative immune markers. We also demonstrated that PF4-dependent proliferation is increased 5–6 days after cardiopulmonary bypass surgery compared to before surgery. These findings suggest that after cardiopulmonary bypass surgery patients possess immune cells that are more responsive to PF4, which may contribute to the immune response that leads to HIT.

1.0 LITERATURE REVIEW

1.1 THE ROLE OF PLATELETS IN HEMOSTASIS AND THROMBOCYTOPENIA

Platelets are critical for protection against exsanguination (Yeaman 2014). They are small discoid cells that do not possess a nucleus and play a direct role in coagulation and thrombus formation (Ghoshal and Bhattacharyya 2014). Platelets are generated in the bone marrow and secreted into the periphery by multinucleated progenitor cells, known as megakaryocytes (Patel, *et al* 2005). Resting platelets contain intracellular granules containing procoagulant factors that induce blood clotting when they become activated (Golebiewska and Poole 2015). Activated platelets interact with collagen and von Willebrand factor on the vascular endothelium to promote platelet aggregation and thrombin generation. Thrombin is required for the production of fibrin, which is required to stabilize blood clots. Although platelets are central to hemostasis, recent studies have highlighted that these cells are versatile in function and can also contribute to inflammation, microbial clearance, and autoimmunity (Rondina and Garraud 2014).

Disorders of platelet function can lead to major bleeding complications such as intracranial hemorrhage (Konkle 2011). Thrombocytopenia, which is defined as a deficiency in the number of platelets within circulation, can lead to tissue bleeding, easy bruising, and delayed blood clot formation. Secondary thrombocytopenia can result from a variety of acquired factors including viral infection, myeloproliferative disorders, autoimmunity, or pharmaceutical therapy (Bussel 2009). According to current laboratory medicine guidelines, a normal circulating platelet count is 150–450x10^9 cells/L (Sekhon and Roy 2006). Mild thrombocytopenia, or a platelet count <150x10^9 cells/L, is transient, asymptomatic, and does not require treatment. Severe thrombocytopenia, or a platelet count of ≤10–20x10^9 cells/L, can lead to life-threatening bleeding complications and may require emergency platelet transfusions. Thrombocytopenia is a well-

studied occurrence in critically ill patients, but estimations of frequency in this population are highly variable (Hui, *et al* 2011). Hospitalized patients exhibit an increased likelihood towards developing thrombocytopenia due to their underlying illnesses and/or multiple drug exposures. (Sajwani and Al Tunaiji 2014).

1.2 DRUG-INDUCED THROMBOCYTOPENIA

Drug-induced thrombocytopenia (DITP) occurs when drugs lead to a decrease in platelet count (Arnold, *et al* 2013). The clinical course of DITP begins within 5–10 days of initial drug exposure and the observed thrombocytopenia has been shown to occur as a result of accelerated platelet clearance or suppressed megakaryocyte function (Aster, *et al* 2009). The incidence of DITP is about 10 persons per million, but this estimate may be higher in elderly or hospitalized patients (van den Bemt, *et al* 2004). Many pathophysiological mechanisms have been shown to cause DITP, including the production of drug-dependent autoantibodies that mediate platelet clearance via the reticuloendothelial system (Curtis 2014). Drugs that can trigger DITP (e.g. sulfonamides, chemotherapy drugs, and antibiotics) alter the conformation of platelet surface proteins to reveal neoepitopes that bind anti-platelet autoantibodies and induce Fc-receptor (FcR)-mediated platelet destruction by phagocytes. The existence of these drug-dependent autoantibodies is typically transient as they disappear from circulation within 1–2 days of drug cessation. Delayed identification of DITP increases the likelihood of developing potentially fatal bleeding complications (Aster and Bougie 2007). Anticoagulant therapy with heparin can lead to the most common immune-mediated DITP, known as heparin-induced thrombocytopenia (HIT) (Franchini 2005).

1.3 HEPARIN-INDUCED THROMBOCYTOPENIA

HIT is a prothrombotic disorder that is initiated by an adverse immune reaction to heparin

(Chong 2003). Heparin is a naturally occurring polysaccharide that is administered to prevent and treat thrombosis. Unfractionated heparin (UFH) (which will be referred to from this point forward as heparin) is a heterogeneous solution of glycosaminoglycans (GAG) of varying molecular weights and degrees of sulfonation (Baglin*, et al* 2006). Heparin remains a widely utilized anticoagulant because of its short half-life, economic feasibility, and ability to be easily neutralized by reversal agents (Kelton and Warkentin 2008). Current estimates suggest that 0.1%–4% of patients administered heparin develop HIT (Seigerman*, et al* 2014).

1.3.1 The pathophysiology of HIT

HIT is diagnosed in critically ill populations, as these patients are typically undergoing anticoagulant therapy (Warkentin 2011a). In addition, patients undergoing major cardiac and orthopedic surgery administered heparin are also at a high risk of developing HIT (Warkentin*, et al* 2000). High levels of platelet activation are associated with major surgical intervention (Oberweis*, et al* 2014, Rinder*, et al* 1991). Platelets contain an abundance of platelet factor-4 (PF4, also known CXCL4) within intracellular granules (Zucker and Katz 1991). PF4 is a 7.8 kDa cationic protein that is secreted from activated platelets as a tetramer bound to a proteoglycan carrier molecule (Huang*, et al* 1982). When free in circulation, PF4 binds to highly sulfated GAGs expressed on endothelial cells within the vasculature (Cella*, et al* 1987). PF4, however, has a higher affinity for binding exogenous heparin (Mayo*, et al* 1995). Because of this tendency to interact, parenteral heparin administration leads to the formation of circulating multimolecular PF4/heparin complexes (Rauova*, et al* 2005). When heparin binds to PF4, conformational changes occur on the PF4 tetramer to reveal neoepitopes (Litvinov*, et al* 2013). In HIT, immunoglobulin G (IgG) antibodies bind these neoepitopes on PF4/heparin (Kelton*, et al* 1994) to form immune complexes (Rauova*, et al* 2005). These immune complexes crosslink FcγRIIa on platelets and

FcγRIa on monocytes to induce Fc-receptor-mediated activation (Warkentin, *et al* 1994). The onset of thrombocytopenia in HIT is primarily a result of widespread antibody-mediated platelet activation, but also enhanced platelet clearance via the reticuloendothelial system (Warkentin, *et al* 2008). HIT is also associated with a thirty-fold increase in the risk of thrombosis (Arepally and Ortel 2006), which occurs when activated platelets and monocytes release procoagulant factors and generate tissue factor to induce thrombin generation and the induction of a hypercoagulable state (Hughes, *et al* 2000).

1.3.2 Clinical hallmarks of HIT

Thrombocytopenia can result from a multitude of clinical factors including illness, drug interactions, or surgical trauma in hospitalized patients (Arnold and Lim 2011). Platelet counts often decrease to a nadir days following surgery (Greinacher and Selleng 2010). Approximately 56% of patients who underwent cardiopulmonary bypass (CPB) surgery experienced a decrease in platelet count by postoperative day 10 (Selleng, *et al* 2010c). An estimated 1% of CPB surgery and 4.9% of major orthopedic surgery patients develop HIT following perioperative exposure to heparin (Warkentin, *et al* 2000). The clinical risk factors associated with HIT include: the type of heparin administered [i.e. heparin is more likely to cause HIT than low molecular weight heparin (LMWH)] (Martel, *et al* 2005), the type of clinical population (LaMuraglia, *et al* 2012), the severity of tissue trauma (Lubenow, *et al* 2010), the duration of heparin exposure (Warkentin and Eikelboom 2007), and the level of pathogenic anti-PF4/heparin IgG antibodies (Nazi, *et al* 2015b). Genetic factors such as polymorphisms of the platelet-expressed Fc-receptor are also associated with an increased susceptibility towards developing HIT-associated thrombosis (Rollin, *et al* 2015). Taken together, these risk factors demonstrate that susceptibility to HIT differs based on clinical and patient-specific genetic factors.

HIT is a "clinical-pathological" disorder as diagnosis depends on concordance between laboratory and clinical assessments (Dutt and Schulz 2013). Early diagnosis is also critical for treating HIT as delays are associated with a 5–10% daily risk of HIT-associated thrombosis, amputation, and death (Greinacher*, et al* 2000). To assist in the clinical diagnosis of HIT, a pretest scoring system known as the 4Ts has been developed for clinicians (Lo*, et al* 2006). The 4Ts system utilizes the four clinical hallmarks of HIT: (1) severity of thrombocytopenia (i.e. ≥50% decrease in platelet count from baseline); (2) the timing of thrombocytopenia (i.e. occurring 5–10 days from the initiation of heparin therapy); (3) the development of thrombosis or other HIT sequelae such as deep vein thrombosis, limb gangrene, or pulmonary embolism; and (4) the probability that other causes are responsible for the thrombocytopenia, since postoperative patients can develop thrombocytopenia as a result of many other factors. The 4Ts system yields an integer score from 0–8, where 0–3, 4–5, and 6–8 are associated with a low, medium, and high pretest probability for HIT. A systematic review of the clinical efficacy of the 4Ts scoring system demonstrates that a low pretest probability is associated with a high negative predictive probability (0.998), but medium and high pretest probabilities are associated with low positive predictive values (0.14 and 0.64, respectively) (Cuker*, et al* 2012). Based on these findings, a low pretest probability can reliably rule out a diagnosis of HIT, but medium-to-high pretest probabilities require further evaluation to rule-in a diagnosis of HIT. Clinical evaluation alone is an inaccurate diagnostic practice as clinical decisions can vary even amongst experienced clinicians (Cuker*, et al* 2010). These authors demonstrate that HIT clinical scoring models are associated with variability between clinicians and a lack of agreement with laboratory testing for HIT.

1.3.3 Laboratory diagnosis of HIT

In conjunction with clinical evaluation, HIT is also tested using laboratory assays. To detect

antibodies that are specific for PF4/heparin, antigenic assays [such as PF4/heparin-dependent enzyme immunoassays (EIA)] are employed by clinical laboratories around the world (Eichler*, et al* 2002). After CPB surgery, up to 70% of patients produce anti-PF4/heparin antibodies in the postoperative period, but a small fraction of these patients develop HIT (Warkentin*, et al* 2013). Antigenic HIT assays, therefore, exhibit a low diagnostic specificity and lead to a high rate of false-positive diagnoses (McFarland*, et al* 2012). The consequences for an incorrect diagnosis of HIT are severe, as these patients are withdrawn from heparin therapy, which increases the likelihood of thrombotic complications (Marler*, et al* 2015). To alleviate the risk of thrombosis, these patients are administered costly alternative anticoagulants, which are associated with a risk of bleeding complications. In addition to antigenic assays, functional HIT assays such as the serotonin-release assay (SRA) are employed to determine the ability for anti-PF4/heparin IgG antibodies to activate platelets (Warkentin*, et al* 2015). The SRA has a high diagnostic specificity (~96%) and can rule in a HIT diagnosis, but is technically demanding and limited in its availability due to the requirement for donor platelets and radioactive isotopes. Functional assays serve as a reliable proxy for HIT as they are able to measure the ability for anti-PF4/heparin IgG antibodies to cause platelet activation (Leo and Winteroll 2003). Discrepancies in the availability and diagnostic capabilities of these laboratory assays continue to pose challenges for HIT diagnosis (Nazi*, et al* 2015a).

1.3.4 Treatment of HIT

Treatment for HIT requires the immediate withdrawal of the patient from heparin therapy (Ahmed*, et al* 2007). Discontinuation of heparin therapy alone, however, does not eliminate the risk of thrombosis as these patients continue to possess a 25–50% of developing blood clots (Wallis*, et al* 1999). In addition to heparin withdrawal, HIT patients are prescribed alternative

anticoagulants such as direct thrombin inhibitors or fondaparinux, which have been shown to be effective in preventing blood clots in patients diagnosed with HIT (Lee and Ansell 2011). Furthermore, a single case study has also demonstrated that B cell-depletion therapy using rituximab can be used to suppress anti-PF4/heparin antibody production in HIT (Schell*, et al* 2013).

1.3.5 Immunological characteristics of HIT

HIT is characterized by an atypical immunological profile that is inconsistent with our understanding of classic antibody responses (Potschke*, et al* 2012). Primary heparin exposure leads to the simultaneous production of anti-PF4/heparin IgG, IgM, and IgA antibodies as IgM precedence does not occur in HIT (Warkentin*, et al* 2009). This is inconsistent with our understanding of classical immune responses, as typical primary antibody responses are characterized by the production of IgM antibodies with primary antigen exposure and the rapid production of IgG antibodies with secondary antigen exposure (Cunningham*, et al* 2014). Additionally, anti-PF4/heparin antibody formation in HIT begins as early as four days after the start of heparin therapy and also precedes the onset of thrombocytopenia (Greinacher*, et al* 2009). The antigenicity of PF4/heparin is characterized by the maximization of antibody binding at equimolar ratios of heparin and PF4, which form charge-neutralized complexes with a molecular weight >670 kDa (Rauova*, et al* 2005). LMWH, in contrast to heparin, forms smaller complexes with PF4, which are less likely to induce anti-PF4/heparin antibody binding and cause HIT (Greinacher*, et al* 2008). HIT is also characterized by the production of two types of anti-PF4/heparin IgG antibodies: (1) antibodies that can cause Fc-receptor-mediated platelet activation and (2) antibodies that cannot cause Fc-receptor-mediated platelet activation (Nazi*, et al* 2015b). A recent study has shown that antibody pathogenicity may be a result of binding to epitopes

expressed on PF4 tetramers bound to GAGs (Cai, *et al* 2015). HIT antibodies do not remain for long periods of time within circulation, as non-pathogenic antibodies become undetectable a median of 85 days and pathogenic antibodies become undetectable a median of 50 days from the onset of HIT (Potschke, *et al* 2012, Warkentin and Kelton 2001). Furthermore, repeat heparin exposure after antibody levels have waned in a patient who has been diagnosed with HIT does not lead to a secondary anti-PF4/heparin antibody response or increase the likelihood of recurrences of HIT (Warkentin and Sheppard 2014). A study into the role of memory B cells in HIT has shown that only 6.7% of cardiac surgery patients who produced anti-PF4/heparin IgG antibodies had PF4/heparin-specific memory B cells in the postoperative period compared to 50% of patients who had memory B cells specific for tetanus toxoid (Selleng, *et al* 2010a). In summary, HIT is characterized by atypical immune features that remain poorly understood in the literature.

1.3.6 Immunobiology of HIT

Greinacher and colleagues proposed that immunological exposure to PF4/polyanion complexes that resemble PF4/heparin can elicit the generation of anti-PF4/polyanion antibodies (Krauel, *et al* 2011). PF4 can bind to other negatively charged molecules such as lipopolysaccharide (Krauel, *et al* 2012) and nucleic acids (Jaax, *et al* 2013) to reveal epitopes that cross-react with anti-PF4/heparin antibodies. PF4 bound to GAGs on endothelial cells, monocytes, and platelets can also bind anti-PF4/heparin antibodies (Rauova, *et al* 2006). Bacterial infection in the absence of heparin exposure is also associated with the production of anti-PF4/heparin antibodies (Greinacher, *et al* 2011). Moreover, studies have demonstrated that HIT can occur in the absence of any heparin exposure, further suggesting that other non-heparin polyanions can alter PF4 to potentiate its immunogenicity and ability to cause HIT (Warkentin, *et al* 2014). Taken together, these studies suggest that exposure to PF4/polyanion complexes leads to a primary anti-

PF4/heparin antibody response and subsequent exposure to heparin may elicit the rapid production of anti-PF4/heparin IgG antibodies that lead to HIT (Krauel*, et al* 2011).

The role of T cells in HIT immunobiology remains controversial. Severe HIT patients possess T cells with highly restricted T cell receptors specific for PF4/heparin (Bacsi*, et al* 1999). Furthermore, both athymic and chimeric mice that lack mature T cells are unable to produce anti-PF4/heparin IgG antibodies (Suvarna*, et al* 2005, Zheng*, et al* 2015). In contrast, murine marginal zone B cells—which can respond to antigens independently of help from T cells—are also required for anti-PF4/heparin IgG antibody production (Zheng*, et al* 2013). Other innate-like B cells, known as B-1 cells, are also critical for the production of anti-PF4/heparin IgM antibodies, which suggest that innate immune mechanisms may contribute to HIT (Krauel*, et al* 2016). Furthermore, patients who underwent liver transplantation surgery can produce anti-PF4/heparin IgG antibodies that activate platelets despite pharmaceutical suppression of T cell function (Bakchoul*, et al* 2014). PF4/heparin has also been shown to induce immune activation via engagement of the pattern recognition receptor, toll-like receptor 4 (TLR4) (Prechel and Walenga 2015). B cell-depletion therapy has also been used as an effective adjunctive therapy for HIT (Schell*, et al* 2013). Dysfunction of immune regulatory mechanisms may also contribute to the onset of HIT (Liu*, et al* 2005), as polyclonal stimulation of human peripheral blood mononuclear cells (PBMCs) and a deficiency of tolerance mechanisms in mice induce the spontaneous generation of anti-PF4/heparin antibodies (Zheng*, et al* 2014). Figure 1 shows a hypothesized biological model that suggests both T cell-dependent and T cell-independent mechanisms contribute to HIT immunobiology.

2.0 INTRODUCTION

2.1 PROJECT RATIONALE

2.1.1 The challenges of diagnosing HIT

The difficulties associated with the laboratory diagnosis of HIT have led to a burden of HIT over-diagnosis in patients who are exposed to heparin (Cuker 2011). Clinical centres utilize antigenic assays to diagnose HIT, but exclusive employment of these assays is associated with a high rate of false-positive results, which can lead to costly and unnecessary interventions (Watson, *et al* 2012). Researchers are to develop new functional assays that do not carry the technical limitations associated with the SRA to diagnose HIT (Padmanabhan, *et al* 2015). In addition to diagnosing HIT after heparin exposure, another method of identification may be in the screening of patients prior to heparin administration for biomarkers that can predict anti-PF4/heparin antibody production, as this can alter the treatment plan prior to heparin administration.

2.1.2 Patient populations at-risk for developing heparin-induced thrombocytopenia

Surgical patients—especially patients undergoing cardiac and orthopedic surgery—have the highest risk of developing HIT (Seigerman, *et al* 2014). In particular, 25%–70% of patients undergoing CPB surgery produce non-pathogenic anti-PF4/heparin antibodies in the postoperative period (Matthai and Cines 2004, Warkentin, *et al* 2013). Extracorporeal circulation, which is used during CPB surgery induces severe inflammation that can resemble the systemic inflammatory response syndrome (Levy and Tanaka 2003). As blood undergoes high shear rates and interacts with the artificial membrane of extracorporeal circulation, both platelets and leukocytes undergo changes in morphology and function (Cella, *et al* 1981, Nguyen, *et al* 1992). CPB surgery can also lead to a dramatic increases in the concentration of circulating PF4 (Cella, *et al* 1981). To reduce the risk of blood clotting, CPB surgery patients are also administered heparin in the intraoperative

period and either heparin or LMWH in the postoperative period (Riess 2005). In surgical populations, it has been hypothesized that the antibody response against PF4/heparin is generated within the intraoperative period since patients are exposed to high concentrations PF4 and heparin in the midst of an inflammatory state (Warkentin*, et al* 2010). Compared to other clinical population, CPB surgery patients are most likely to generate an antibody response against PF4/heparin in the perioperative period (Warkentin*, et al* 2013); therefore, we decided that this surgical population would be most informative in studying the anti-PF4 immune response before and after exposure to heparin therapy.

2.1.3 Utilizing the immune response prior to heparin exposure to predict HIT onset

Since the first clinical description of HIT (Natelson*, et al* 1969), investigators have investigated its pathophysiology, clinical features, laboratory diagnostics, and immunological characteristics; however, less progress have been made in the understanding of the immunobiology that underlies anti-PF4/heparin antibody generation in HIT. A recent hypothesis into the possible biological underpinnings of HIT antibody production suggests that the likelihood of developing HIT depends on the repertoire of immune cells that exist prior to heparin exposure (Krauel*, et al* 2011). PF4 can undergo conformational changes that permit anti-PF4/heparin antibody cross-reactivity when bound to non-heparin polyanions like lipopolysaccharide or GAGs (Prechel and Walenga 2013). Based on this finding, researchers have proposed that exposure to PF4/polyanion complexes in the absence of heparin exposure may "prime" the PF4/heparin-specific immune response to become activated after heparin exposure (Krauel*, et al* 2011). Studies, however, have not evaluated whether the immune response prior to heparin infusion underlies susceptibility to HIT surgical patients. A screening assay that can identify patients prior to heparin exposure who possess an increased risk for developing HIT will help to prevent the devastating complications

associated with this iatrogenic disorder.

2.2 RESEARCH HYPOTHESIS

Based on the premise that immunity to PF4/heparin can be "primed" by exposure to PF4/polyanion complexes, we hypothesize that a subset of CPB surgery patients who possess circulating immune cells that response to stimulation with PF4 are more likely to produce anti-PF4/heparin antibodies following intraoperative heparin exposure than patients who do not possess these cells.

As of yet, a biomarker has not been discovered that can predict the onset of HIT prior to heparin exposure. This research serves as the first known attempt to utilize immune assays prior to heparin exposure to predict surgical patients who may be at an increased risk for developing HIT.

2.3 RESEARCH OBJECTIVES

We utilized a prospective cohort study design to investigate the preoperative PF4-specific immune response in CPB surgery patients. We employed two immunological assays to investigate the role of preoperative PF4-specific immunity in HIT susceptibility in surgical patients: (1) ^{3}H-thymidine uptake assay, which measures cellular proliferation in response to exogenous stimuli, and (2) a PBMC ELISPOT, which quantifies the number of antigen-specific antibody-secreting cells (ASC) in circulation. The objectives of the current study are multifold: (1) To determine whether the proliferation of PBMCs to PF4 prior to CPB surgery is associated with anti-PF4/heparin antibody production after surgery; (2) To determine whether a history of heparin exposure or major surgery can identify patients who are more likely to possess PBMCs that proliferate in response to PF4 prior to surgery; (3) To determine whether the preoperative frequency of PF4-specific ASCs is associated with postoperative class-specific anti-PF4/heparin

antibody production; (4) To determine whether patients that have PBMCs that undergo higher levels of PF4-dependent proliferation after CPB surgery compared to before surgery. We also evaluated whether stimulation with PF4/heparin can predict postoperative anti-PF4/heparin antibody production; however, since our hypothesis addresses the ability for pre-heparin immunity (i.e. PF4 altered by endogenous polyanions) to predict postoperative antibody production, we focused our discussion on PF4 alone. The findings of this study will provide insight into how the preoperative immune response to PF4 may identify patients who are at risk of developing HIT after CPB surgery.

3.0 MATERIALS AND METHODS

3.1 PATIENT RECRUITMENT, DATA EXTRACTION, AND CLINICAL DEFINITIONS

Patients recruited into study were scheduled to undergo cardiac surgery at the Hamilton General Hospital in Hamilton, Ontario. Inclusion criteria included patients ≥18 years of age who scheduled to undergo major cardiac surgery on CPB. Blood samples were collected before surgery (day 0) and once 4–8 days after surgery. Whole blood collected before surgery (day 0) was drawn into sterile ACD vacutainers and processed within two hours of collection. Serum and plasma samples were drawn once between days 4–8 after surgery, separated, and frozen at –80°C until use. Patients were recruited in conjunction with assistance from the cardiac investigation team at the Hamilton Generation Hospital. The majority of patients included in the study [39 out of 44 patients (89%)] were confirmed to have been administered intraoperative heparin and protamine sulfate for anticoagulation and neutralization, respectively. Overall, fifty-six patients were recruited into the current study, but forty-four patients were included in the final analysis. Two patients (n=2) were excluded because they did not undergo surgery on CPB, one patient (n=1) did not have a preoperative blood sample collected, seven patients (n=7) did not have a postoperative blood sample collected, and two patients (n=2) were excluded because they were on immunosuppressant therapy at the time of recruitment. All protocols and patient materials were approved by the HiREB.

We evaluated the history of prior heparin exposure as “definite” if heparin use was documented in the patient’s medical records, as “possible” if there had been at least one previous hospitalization during which treatment with heparin would have been likely, and as “unlikely” if there had been no hospitalization or hospitalizations during which heparin therapy would have been unlikely. We also evaluated previous surgical trauma as “major surgery”, which was defined

as a documented procedure in which a body cavity was entered, an organ was removed, in-patient period was >4 days, or there was a high likelihood that postoperative prophylactic anticoagulants were administered. All data extraction protocols and materials were approved by the HiREB.

3.2 PURIFICATION OF HUMAN PLATELET-DERIVED PF4

Human platelet-derived PF4 was obtained from pooled platelets, which were lysed and purified according to previously described methods (Levine and Wohl 1976). Pooled platelet-derived PF4 was further purified using reverse-phase chromatography on a HPLC fitted with a C4 column. The stationary phase of the C4 column is ideal for separation of proteins with a molecular weight >10 kDa and reverse-phase HPLC using a C4 column can decrease the levels of lipopolysaccharide (LPS) from protein samples (Dudley*, et al* 2003). Purified PF4 was then lyophilized and suspended in sterile 1.5 M sodium chloride buffer. LPS was removed using endotoxin-specific syringe filers until the endotoxin levels were <0.5 EU/mL. A chromogenic LAL endotoxin assay was utilized to quantify the endotoxin levels and a BCA was used to determine the concentration of PF4 protein. The purity of the pooled platelet-derived PF4 was determined using size-dependent SDS-PAGE. We also evaluated the antigenicity of the pooled platelet-derived PF4 using an in-house PF4/heparin-dependent IgG-specific EIA. To calculate the stoichiometric ratios of PF4 and heparin we utilized previously published estimates of heparin specific activity at 140 U/mg, a heparin molecular weight of 15 kDa, and a PF4 molecular weight of 31.8 kDa (Chudasama*, et al* 2010).

3.3 PBMC ISOLATION

PBMCs were isolated from whole blood via density centrifugation using histopaque-1077 (i.e. 400g for 30 minutes). PBMCs were washed once in PBS supplemented with 10% FBS and once in PBS supplemented with 10% FBS with heparin to prevent the aggregation of cells. Our

laboratory has demonstrated that the addition of heparin during this stage does not affect the results of the proliferation assay. Both washes were centrifuged at 370g for 10 minutes. PBMCs were then suspended in serum-free AIM-V cell culture media and counted using a hemocytometer. Serum-free AIM-V cell culture media was used in this study because it has been shown to reduce background and optimize signal in ^{3}H-thymidine proliferation assays (Kaldjian*, et al* 1992).

3.4 ^{3}H-THYMIDINE UPTAKE ASSAY

PBMCs ($1x10^{6}$/mL) were cultured in AIM-V cell culture media with various proteins-of-interest: (1) human platelet-derived PF4 (50 μg/mL), (2) human platelet-derived PF4 (50 μg/mL) and heparin (0.25 U/mL), and (3) Resiquimod (R848) (2.5 μg/mL) and interleukin (IL)-2 (10 ng/mL). R848 and IL-2 were utilized as positive controls because both of these compounds have been shown to be potent activators of cells within the PBMC pool (Jahnmatz*, et al* 2013). To form PF4/heparin complexes, PF4 and heparin were incubated for 20 minutes in AIM-V cell culture media at room temperature. PBMCs were also cultured in AIM-V cell culture media alone to serve as a negative control. Each condition was plated in six replicates in 96 well sterile tissue culture plates. Plates were incubated for six days at 37°C and 5% CO_2. On day six, each well was pulsed with ^{3}H-thymidine (0.5 μCi/well) and incubated for an additional 16 hours at 37°C and 5% CO_2. PBMCs were then lysed onto glass fibre filter mats using a cell harvester. Filter mats were dried overnight at room temperature and the following day the mats were placed in vacuum-sealed plastic envelopes. To amplify the radioactive signal, β-scintillate was added to each vacuum-sealed envelope. Mats were then placed in a luminescence counter and β-radioactivity from each mat was quantified in counts per minute (CPM). Proliferation was quantified as SI, which was calculated by dividing the average CPM of the protein-of-interest condition by the average CPM of the negative control condition (i.e. media alone).

3.5 PF4-SPECIFIC ELISPOT ASSAY

As described previously, PBMCs were isolated from 30 mL of whole blood collected into sterile ACD vacutainers. PBMCs were then plated at a concentration of $2x10^6$ cells/mL in AIM-V culture media with R848 (2.5 µg/mL) and IL-2 (10 ng/mL) in sterile twenty-four well tissue culture plates. These plates were incubated for six days at 37°C and 5% CO_2. Both published findings (Jahnmatz*, et al* 2013) and previous experiments completed by our laboratory demonstrated that six days of stimulation optimized B cell activation for the ELISPOT assay. PVDF multiscreen MAIPS4510 plates were hydrated with 70% ethanol and sterile H_2O five days after the PBMC isolation. The hydrated plate was then coated with anti-human IgG, IgM, and IgA antibodies and PBS alone. Plates were incubated overnight at 2–3°C. Six days after the PBMC isolation the antibodies were decanted from the plate, washed, and blocked with PBS supplemented with 3% BSA. The plate was then incubated for 2 hours at 37°C and 5% CO_2. On the same day, stimulated PBMCs were collected and washed three times in AIM-V cell culture media (370g for 10 minutes). Following the 2-hour incubation period, the plate was decanted and washed one time with PBS. Washed PBMCs were then added to the plate at 200,000 cells/well for antigen-specific and total IgA antibody wells and 10,000 cells/well for total IgG and IgM antibody wells. Stimulated PBMCs were then plated in four replicates for each antibody condition. PBMCs were also added to wells coated with PBS alone. AIM-V cell culture media alone was added to antibody-coated wells in duplicates. Plates were then incubated for 19 hours at at 37°C and 5% CO_2. The following day, PBMCs were decanted from the plate and the plate was washed with a solution of PBS supplemented with 0.5% Tween-20. Next, human platelet-derived PF4 and biotinylated human platelet-derived PF4 were diluted to 25 µg/mL and combined in PBS supplemented with 0.5% BSA. Employment of a mixture of platelet-derived and biotinylated PF4 has been shown to reduce

the number of background spots in an ELISPOT assay (Schulze*, et al* 2014). Two conditions were then prepared: (1) PF4 alone, which does not contain heparin and (2) PF4/heparin, which contains 20 U/mL heparin combined with 25 μg/mL of the PF4 solution. This ratio of PF4-to-heparin corresponds to those used in the PF4/heparin-dependent EIA. Moreover, biotinylated IgG, IgM, and IgA were combined with PBS supplemented with 0.5% BSA and filtered through 0.2 μm syringe filters. The two PF4 solutions and three biotinylated antibody solutions were then added to the plate. Once added, the plate was incubated for 2 hours at 37°C. After the 2-hour incubation, the plate was washed with PBS supplemented with 0.5% Tween-20. Next, streptavidin conjugated to alkaline phosphatase was added to the plate and incubated for in the dark one hour at room temperature. After the incubation period, the plate is washed and the substrate, BCIP was added to the plate. The plate was incubated in the dark for 30 minutes at room temperature and washed multiple times with distilled H_2O. The plate was then allowed to air dry overnight at room temperature. The number and size of spots were then quantified using the BioSys Bioreader 6000. The frequency of antigen-specific B cells was calculated first by subtracting the number of spots in the no coat wells from the number of spots in the antigen-specific wells. Next, we divided the mean number of antigen-specific spots by the mean number of corresponding class-specific antibody spots and multiplied by 1000.

3.6 POLYSPECIFIC PF4/POLYANION-DEPENDENT EIA

Utilized to detect *in vivo* antibodies capable of binding to PF4 bound to anions such as polyvinyl sulfate. Assays were performed according to manufacturer's guidelines (GTI, Waukesha, WI). For the detection of anti-PF4/polyanion polyspecific antibodies, a positive result was classified by an $OD_{405} \geq 0.40$ and a negative result was classified as <0.40. Standard positive and negative controls were included on each plate.

3.7 ISOTYPE-SPECIFIC PF4/HEPARIN-DEPENDENT EIA

In vivo class-specific anti-PF4/heparin antibodies were measured with a PF4/heparin-dependent EIA specific for IgG, IgM, and IgA antibodies. Ninety-six well Maxisorp plates were coated with 60 µg/mL of PF4 and 1 U/ml of heparin and underwent a one-hour incubation at room temperature. After washing, the plates were blocked with 3% BSA for two hours at room temperature. Patient or control sera, which were diluted at 1:50, were added to wells and incubated for one hour. After washing, alkaline phosphatase conjugated anti-human IgG, IgM, or IgA were added to wells at a 1:4000 dilution followed by the addition of the substrate, p-NPP to detect PF4/heparin-bound anti-human IgG, IgM, or IgA antibodies. UV absorbance was then measured using a microplate photometer and the OD_{405} was used to assess the presence or absence of *in vivo* anti-PF4/heparin antibodies. For the detection of anti-PF4/heparin IgG and IgM antibodies, a positive result was classified by an $OD_{405} \geq 0.45$ and a negative result was classified by an $OD_{405} < 0.45$. For the detection of anti-PF4/heparin IgA antibodies, a positive result was classified by an $OD_{405} \geq 0.35$ and a negative result was classified by an $OD_{405} < 0.35$. These OD cut-offs were defined previously in the literature and represent two standard deviations above the mean OD (Nazi, *et al* 2015a). Standard positive and negative control samples were included on each test plate.

3.8 PROTEINASE K DIGESTION OF PF4

Human platelet-derived PF4 was incubated overnight at room temperature with proteinase K in 1.5 M sodium chloride buffer. Proteinase K degrades amino bonds within polypeptide chains and was used to digest purified human platelet-derived PF4 in this study.

3.9 STATISTICAL ANALYSIS

For all variables measured, normality of data distributions was tested using the Shapiro-Wilk

test. If a distribution was classified as non-normal, non-parametric statistical analyses were performed (i.e. Wilcoxon rank-sign test for paired samples and Spearman's ρ to assess correlation, and Kruskal-Wallis for analysis of variance). For any data distributions that were positively skewed, we used geometric mean as a measure of central tendency. If variables were determined to follow a normal distribution, we utilized mean as a measure of central tendency and we used parametric analyses to make statistical inferences. For statistical analyses, an alpha value of 0.05 was utilized to assign statistical significance. All statistical analyses were performed using SPSS (IBM) statistical software. All graphs were completed using Graphpad Prism 6.

4.0 RESULTS

4.1 VALIDATION OF THE PF4-DEPENDENT PROLIFERATION ASSAY

4.1.1 PF4 is required to induce proliferation of PBMCs

We determined whether human platelet-derived PF4 was required to induce the *in vitro* proliferation of PBMCs isolated from a healthy volunteer. To complete this objective, PF4 was digested using proteinase K and compared to non-digested PF4 on a protein gel (Figure 2a). We utilized proteinase K to digest PF4 to ensure that PF4-dependent PBMC proliferation is not due to the presence of non-protein contaminants (i.e. LPS). Next, digested and non-digested PF4 were included in a ^{3}H-thymidine uptake assay. We demonstrated that PBMC proliferation decreased with digested PF4 compared to non-digested PF4 [mean non-digested PF4 proliferation (in CPM) vs. mean digested PF4 proliferation (in CPM) ± SEM of six replicates: 6612.4 ± 758.2 vs. 481.9 ± 57.9] (Figure 2b). To assess proliferation relative to the media control, we also calculated SI for non-digested PF4 and digested PF4, which were 9.2 and 0.64, respectively. In addition, digested PF4 combined with 0.25 U/mL of heparin also lead to a decrease in proliferation compared to non-digested PF4 complexed with 0.25 U/mL of heparin [mean non-digested PF4/heparin proliferation

(in CPM) vs. mean digested PF4/heparin proliferation (in CPM) ± SEM of six replicates: 9012.9 ± 948.9 vs. 422.3 ± 63.5] (Figure 2b). The SIs for non-digested PF4/heparin and digested PF4/heparin were 12.5 and 0.56, respectively. Proliferation was not affected when PBMCs were incubated in media alone in either the non-digested or digested culture plates [mean media proliferation (in CPM) vs. mean media proliferation (in CPM) ± SEM of six replicate wells: 718.9 ± 78.0 vs. 757.50 ± 68.6] (Figure 2b). To ensure that PBMCs maintained viability in both culture plates, we also incubated the PBMCs with R848 and IL-2, which can induce global proliferation of PBMCs [mean R848 and IL-2 proliferation in non-digested culture plates (in CPM) vs. mean R848 and IL-2 proliferation in digested culture plates (in CPM) ± SEM of six replicate wells: 17386 ± 782.3 vs. 15791 ± 952.2] (data not shown). In summary, results from these experiments demonstrated that the proliferation of PBMCs was primarily dependent on the presence of intact PF4 and not additional non-protein contaminants such as LPS.

4.1.2 Heparin modifies PF4-dependent proliferation

To address whether heparin had an effect on the PF4-dependent proliferation response, we incubated PBMCs from healthy volunteers with PF4 complexed with various concentrations of heparin. We previously demonstrated in our laboratory that the majority of healthy volunteers did not possess PBMCs that proliferate in response to stimulation with PF4; however, a subset of these healthy volunteers did possess PBMCs that were responsive to PF4. We demonstrated in two of these healthy volunteers (n=2) that heparin was able to modify the PF4-dependent proliferation response. In the absence of heparin, we demonstrated that PF4 at a concentration of 50 μg/mL induced PBMC proliferation [mean PF4 alone proliferation (in SI) ± SEM: 3.1 ± 0.36] (Figure 3). PF4-dependent proliferation increased when 0.25 U/mL of heparin was added to the PF4 (50 μg/mL) [mean PF4 combined with 0.25 U/mL heparin proliferation (in SI) ± SEM: 8.4 ± 0.36]. In

contrast, proliferation decreased when 2.5 U/mL of heparin was added to PF4 (50 μg/mL) [mean PF4 combined with 2.5 U/mL heparin proliferation (in SI) ± SEM: 0.97 ± 0.11]. Taken together, these findings demonstrated that PF4 was able to induce proliferation and that the addition of heparin was able to modify the PF4-dependent proliferation response of PBMCs.

4.2 ASSESSING THE RELATIONSHIP BETWEEN PREOPERATIVE PF4-DEPENDENT PROLIFERATION AND POSTOPERATIVE ANTI-PF4/HEPARIN ANTIBODY PRODUCTION

4.2.1 Baseline demographic and surgical characteristics of patient cohort

We analyzed forty-four patients at two time points: before and 4–8 days after CPB surgery. Table 1 demonstrates the baseline demographic and surgical characteristics of the cardiopulmonary bypass patient cohort included in the final analysis. Prior to CPB surgery, patients had mean platelet and leukocyte counts within the respective reference intervals, which suggests that these patients did not suffer from platelet or lymphoproliferative disorders at the time of recruitment. Table 1 also shows the preoperative, intraoperative, and postoperative characteristics of the patient cohort. Prior to CPB surgery, a majority of patients [42 out of 44 patients (95.0%)] were confirmed to have undergone cardiac catheterization, which is suggestive of exposure to a heparin-coated catheter, but this exposure to heparin could not be confirmed by medical chart review. With regards to intraoperative characteristics, all patients included in the final analysis were confirmed to have undergone surgery on CPB. In addition, the majority of patients were confirmed to have been administered heparin during surgery and all patients were confirmed to have been administered LMWH for thromboprophylaxis for a mean length of time of six days.

4.2.2 Rates of preoperative and postoperative anti-PF4/heparin antibody production

CPB is known to induce the postoperative production anti-PF4/heparin antibodies in up to 70% of patients (Warkentin*, et al* 2013). Table 2 shows the number of patients who tested positive for anti-PF4/heparin antibodies before and after CPB. Using the PF4/polyanion-dependent EIA, 25.0% and 31.8% of patients tested positive for anti-PF4/heparin antibodies before and 4–8 days after surgery, respectively. We also demonstrated that after surgery, 15.9%, 29.5%, and 15.9% of patients produced anti-PF4/heparin IgG, IgM, and IgA antibodies, respectively. Compared to the other two antibody classes, the highest proportion of patients [13 out of 44 (29.5%)] produced anti-PF4/heparin IgM antibodies in the 4–8-day period after surgery. Within the 4–8-day postoperative collection period, the majority of samples were collected on day 5 [20 out of 44 (45.5%)]. Furthermore, the proportion of patients who tested positive for anti-PF4/heparin antibodies according to postoperative day were: day 4 [0 out of 8 (0.00%)], day 5 [3 out of 20 (15.0%)], day 6 [5 out of 9 (55.6%)], day 7 [4 out of 5 (80%)], and day 8 [2 out of 2 (100%)]. These results demonstrate that samples collected on day 6, 7, and 8 had a greater likelihood of being positive for anti-PF4/heparin antibodies; however, further conclusions about postoperative antibody production cannot be made since samples were not collected from patients on sequential days in the postoperative period. Table 3 shows the mean levels of anti-PF4/heparin antibodies both before and 4–8 days after CPB surgery. This table demonstrates that the mean of anti-PF4/heparin antibody levels was negative both before and 4–8 days after CPB surgery. Furthermore, the mean of class-specific anti-PF4/heparin antibody levels before and after were also negative, which suggests that the majority of this cohort of CPB surgery patients did not produce high levels of anti-PF4/heparin antibodies postoperatively.

4.2.3 Levels of preoperative proliferation in CPB surgery patient cohort

We measured PF4- and PF4/heparin-dependent proliferation in this cohort of patients prior

to CPB surgery. Figure 4 demonstrates no difference in the levels of PF4-dependent and PF4/heparin-dependent proliferation within this cohort of patients prior to surgery [GM PF4 alone proliferation (in SI) vs. GM PF4 combined with 0.25 U/mL heparin proliferation (in SI) ± geometric SEM: 3.54 ± 1.2 vs. 3.43 ± 1.2, $P = 0.93$]. To demonstrate the proliferative capacity of these cells, we also incubated the PBMCs with R848+IL-2 (positive control) [GM R848+IL-2 proliferation (in SI) ± geometric SEM: 25.9 ± 1.4] (data not shown). Figure 5 shows a strong positive correlation between PF4- and PF4/heparin-dependent proliferation in this patient cohort prior to surgery (Spearman's $\rho = 0.869$, $P < 0.0001$). Taken together, these findings demonstrate that the addition of heparin did not impact PF4-dependent proliferation in this cohort of patients prior to CPB surgery.

4.2.4 Preoperative PF4-dependent proliferation is not associated with postoperative anti-PF4/heparin antibody production in this cohort of CPB surgery patients

We investigated whether preoperative PF4-dependent proliferation is associated with postoperative anti-PF4/heparin antibody production. Our findings show that preoperative PF4-dependent proliferation was not associated with the postoperative production of anti-PF4/heparin antibodies [Spearman's ρ (95% CI) = –0.02 (–0.32, 0.28), $P = 0.91$] (Figure 6a). Preoperative PF4/heparin-dependent proliferation is also not associated with the postoperative production of anti-PF4/heparin antibodies [Spearman's ρ (95% CI) = 0.03 (–0.27, 0.32), $P = 0.86$] (data not shown). Based on the current sample size and the limits of the 95% CI, we conclude that preoperative PF4-dependent proliferation is unable to predict postoperative polyspecific anti-PF4/heparin antibody production within this patient cohort.

We also assessed whether preoperative PF4-dependent proliferation is associated with the postoperative production of class-specific (i.e. IgG, IgM, or IgA antibodies) anti-PF4/heparin

antibodies. Preoperative PF4-dependent proliferation demonstrated a weak negative association with postoperative anti-PF4/heparin IgG antibody production [Spearman's ρ (95% CI) = –0.31 (–0.56, –0.02), P = 0.04] (Figure 6b). Investigations into whether preoperative PF4-dependent proliferation could explain variation in postoperative anti-PF4/heparin IgG antibody production demonstrated a weak fit with a linear model (R^2 = 0.07). Our findings also demonstrated that PF4-dependent proliferation was not associated with the postoperative production of anti-PF4/heparin IgM or IgA antibodies [IgM: Spearman's ρ (95% CI) = –0.04 (–0.33, 0.26), P = 0.78; IgA: Spearman's ρ (95% CI) = –0.05 (–0.34, 0.25), P = 0.73] (Figure 6c–d). Moreover, PF4/heparin-dependent proliferation was not associated with the postoperative production of anti-PF4/heparin IgG, IgM, or IgA antibodies [IgG: Spearman's ρ (95% CI) = –0.20 (–0.47, 0.10), P = 0.18; IgM: Spearman's ρ (95% CI) = 0.03 (–0.27, 0.32), P = 0.83; IgA: Spearman's ρ (95% CI) = –0.07 (–0.36, 0.23), P = 0.65] (data not shown). Based on the sample size and the limits of the 95% CI, we conclude that preoperative PF4-dependent proliferation cannot predict postoperative class-specific anti-PF4/heparin antibody production within this patient cohort.

In addition to analyzing the association between preoperative proliferation and postoperative antibody production for the entire patient cohort, we qualitatively evaluated this relationship from the perspective of individual patients. Figure 7a–d demonstrates the relationship between preoperative PF4-dependent proliferation (measured in SI) and postoperative polyspecific and class-specific anti-PF4/heparin antibody levels for individual patients. The majority of patients in this cohort possessed a low-magnitude PF4-dependent proliferation response; however, two patients had PF4-dependent SIs of 53.0 and 45.8 preoperatively. These two patients also produced the highest levels of anti-PF4/heparin IgM antibodies (i.e. OD_{405} = 2.38 and 1.61). A similar trend was also observed with preoperative PF4/heparin-specific proliferation and postoperative anti-

PF4/heparin IgM antibody levels (data not shown). Since these are anti-PF4/heparin IgM antibodies, they are not able to cause HIT and were therefore not evaluated for platelet-activating abilities using a functional assay. We also evaluated the relationship between the levels of anti-PF4/heparin antibodies both before and after surgery on a patient-specific level (Figure 8a–d). In contrast to preoperative PF4-dependent proliferation, patients who possessed the highest levels of preoperative anti-PF4/heparin antibodies were not the same patients who produced the highest levels of postoperative anti-PF4/heparin antibodies. Based on these findings, it seems as through the two patients who had a high preoperative PF4-dependent proliferation response were more likely to produce postoperative anti-PF4/heparin IgM antibodies. Further conclusions cannot be made since the majority of patients had PBMCs that did not proliferate strongly in response to preoperative stimulation with PF4.

4.3 ASSESSING THE RELATIONSHIP BETWEEN A HISTORY OF HEPARIN EXPOSURE OR MAJOR SURGERY AND PREOPERATIVE PROLIFERATION

4.3.1 History of heparin exposure and preoperative PF4-dependent proliferation

In this cohort of forty-four patients, we investigated whether a history of previous heparin exposure is associated with higher levels of PF4-dependent proliferation. The purpose of this analysis was to further describe this heterogeneous patient cohort and to determine whether we could identify a clinical marker that was associated with PF4-dependent proliferation. We categorized patients into three groups based on the likelihood of a history of heparin exposure. Table 4 shows that preoperative PF4-dependent proliferation is higher in patients who have had a definite previous exposure to heparin compared to patients who had a possible or unlikely heparin exposure, but this difference is not statistically significant. Preoperative polyspecific or class-specific anti-PF4/heparin antibody levels also did not differ significantly based on the likelihood

of previous heparin exposure (Table 4). Due to the small sample size of patients within the category of "definite" heparin exposure, however, it is difficult to make conclusive statements about the effect of previous heparin exposure on preoperative PF4-dependent proliferation.

4.3.2 History of surgical trauma and preoperative PF4-dependent proliferation

We also investigated whether a history of surgical tissue trauma of varying severity is associated with increased levels of PF4-dependent proliferation. Major surgical trauma has been shown to be an independent predictor of HIT (Lubenow*, et al* 2010), and so the purpose of this analysis was to determine whether the severity of tissue trauma was related to the preoperative PF4-dependent proliferation response. To do this, we categorized patients into three groups based on whether each patient had undergone a surgical procedure classified as "major" or "minor", or had not undergone a surgical procedure according to their medical records (Table 5). Table 6 shows that preoperative PF4-dependent proliferation and preoperative anti-PF4/heparin antibody levels did not differ based on the history of surgical tissue trauma.

4.4 MEASURING THE RELATIONSHIP BETWEEN PREOPERATIVE FREQUENCY OF PF4-SPECIFIC ASCs AND POSTOPERATIVE ANTI-PF4/HEPARIN ANTIBODY PRODUCTION

4.4.1 Preoperative frequency of PF4-specific ASCs and class-specific postoperative anti-PF4/heparin antibody levels

In a subset of the patients recruited into this study (n=5), we performed an ELISPOT assay to quantify the frequency of PF4-specific ASCs in circulation prior to surgery and to identify whether this frequency of was associated with postoperative anti-PF4/heparin antibody production. Based on the small sample size, we determined that the preoperative frequency of PF4-specific IgM- or IgG-secreting ASCs are not associated with levels of anti-PF4/heparin IgM or

IgG antibodies after CPB surgery [IgM: Spearman's ρ (95% CI) = 0.30 (–0.79, 0.93), P = 0.683; IgG: Spearman's ρ (95% CI) = –0.21 (–0.92, 0.83), P = 0.600] (Figure 9a–b). Based on the small sample size and large range of correlation coefficients covered within the 95% CIs, we are unable to make convincing conclusions regarding the relationship between these two variables until sample size is increased. In addition, the preoperative frequency of PF4/heparin-specific IgM- or IgG-secreting ASCs were not associated with the levels of anti-PF4/heparin IgM or IgG antibodies after CPB surgery [IgM: Spearman's ρ (95% CI) = –0.21 (–0.92, 0.83), P = 0.600; IgG: Spearman's ρ (95% CI) = –0.87 (–0.99, 0.05), P = 0.03] (data not shown). Our small data set suggests that no relationship exists between preoperative ASC frequency and postoperative anti-PF4/heparin antibody levels, but future investigations with a larger sample size are required to make meaningful conclusions.

4.5 MEASURING THE EFFECT OF CPB SURGERY ON PF4-DEPENDENT AND PF4/HEPARIN-DEPENDENT PROLIFERATION

4.5.1 PBMC proliferation is enhanced in the postoperative period after cardiopulmonary bypass surgery

We investigated PF4-dependent and PF4/heparin-dependent proliferation in this cohort of patients before and after CPB surgery (n=12). Figure 10 shows that after CPB surgery patients exhibit higher levels of PF4-dependent proliferation compared to their preoperative levels of PF4-dependent proliferation [GM postoperative PF4 alone proliferation (in SI) vs. GM preoperative PF4 alone proliferation (in SI) ± geometric SEM: 23.7 ± 1.3 vs. 6.9 ± 1.5, P = 0.009] (Figure 10). In the postoperative period, these patients also had a higher PF4/heparin-dependent proliferation response compared to the preoperative time-point, but this comparison did not reach statistical significance [GM postoperative PF4 combined with 0.25 U/mL heparin proliferation (in SI) vs.

GM preoperative PF4 combined with 0.25 U/mL heparin proliferation (in SI) ± geometric SEM: 19.1 ± 1.4 vs. 8.18 ± 1.5, $P = 0.064$] (Figure 13). The mean proliferation in media alone did not increase after CPB surgery when compared to preoperative levels of proliferation in media alone [mean postoperative media alone proliferation (in CPM) vs. mean preoperative media alone proliferation (in CPM) ± SEM: 572.5 ± 85.9 vs. 624.7 ± 115.9, $P = 0.719$] (data not shown). An important limitation of these findings are the lack of a non-PF4 protein to induce proliferation and a group of patients who did not undergo surgery on CPB. Taken together, these findings preliminarily suggest that proliferation of PBMCs following *in vitro* stimulation with PF4 increases in the period following CPB surgery.

5.0 DISCUSSION

HIT is an immune-mediated prothrombotic disorder that develops as a consequence of heparin therapy (Warkentin 2011b). Recent advances in the understanding of HIT immunobiology have suggested that the immune cell repertoire prior to heparin exposure may underlie susceptibility to HIT following heparin exposure (Krauel*, et al* 2011). In this study, we investigated whether the immune response to PF4 before heparin exposure is associated with the likelihood of developing the HIT immune response after surgery-associated heparin exposure.

First, we demonstrated that *in vitro* stimulation with human platelet-derived PF4 is able to induce the proliferation of PBMCs derived from patients before CPB surgery. Previous findings from our laboratory have shown that PF4-dependent proliferation of PBMCs is higher in HIT patients in comparison to healthy volunteers. PF4 is a chemokine that is versatile in function and has been shown to affect innate and adaptive immune cell function (Kasper and Petersen 2011). PBMCs are comprised of a heterogeneous pool of immune cells, including B cells, T cells, monocytes, and dendritic cells (Autissier*, et al* 2010). Studies have demonstrated that *in vitro* stimulation with PF4 suppresses the proliferation of isolated $CD4^+$ T cells (Fleischer*, et al* 2002) but stimulates the proliferation of $CD4^+CD25^+$ T cells (Liu*, et al* 2005). Furthermore, recent findings have demonstrated that PF4/heparin is readily phagocytosed by dendritic cells (Joglekar*, et al* 2015) and PF4/heparin bound to complement can engage complement receptors (i.e. CR2) on human B cells (Khandelwal*, et al* 2016). The precise role of T cells and other immune cells in HIT remains poorly understood (Bakchoul*, et al* 2014).

Preliminary research from our laboratory using EdU to label newly synthesized DNA in PBMC cultures stimulated with PF4 suggests that surface expression of CD14 increases alongside the synthesis of DNA. CD14 is expressed on monocytes and dendritic cells, which comprise an

estimated 20% and 1.5% of human PBMCs, respectively (Autissier, *et al* 2010). *In vitro* stimulation with PF4 can facilitate monocyte chemotaxis (Osterman 1982) and the differentiation of monocytes into macrophages and dendritic cells (Fricke, *et al* 2004, Scheuerer 2000, Xia and Kao 2003). Human peripheral blood monocytes were originally classified as non-proliferating cells (van Furth, *et al* 1979); however, recent evidence demonstrates that a subset of peripheral blood monocytes can undergo proliferation following *in vitro* stimulation with M-CSF (Clanchy, *et al* 2006). Monocytes also do not proliferate as they differentiate into dendritic cells (Ardeshna, *et al* 2000). Preliminary studies from our laboratory suggest that CD14 expression increases following stimulation of human PBMCs with PF4, but the ability for human-derived monocytes to undergo proliferation remains controversial. Consistent with a role for CD14-expressing cells in the PF4-dependent proliferation response, PF4/heparin complexes have been shown to engage TLR4 on human PBMCs and induce immune activation via IL-8 secretion (Prechel and Walenga 2015). Future studies of additional cell surface markers are required to determine the precise phenotype of the PBMC subset(s) that undergo proliferation in response to *in vitro* stimulation with PF4.

At a PF4-to-heparin ratio (PHR) = 13:1 (i.e. 0.25 U/mL heparin added to 50 μg/mL PF4) proliferation was higher than that observed in PF4 alone. Studies have not investigated the effect of PF4 on proliferation, but murine studies of PF4/heparin immunogenicity demonstrate maximal anti-PF4/heparin antibody generation with a PHR of 20:1 and 10:1 (Suvarna, *et al* 2007). Heparin also enhances the ability for cationic proteins including PF4 to induce IL-12 secretion by dendritic cells (Chudasama, *et al* 2010) and endocytosis of PF4 by dendritic cells is maximized at a PHR = 7:1 (Joglekar, *et al* 2015). The ability for PF4/heparin to activate TLR4 on human PBMCs is maximized at PHR of 12.5:1, which is consistent with the PHR that we employed in our studies.

The ability of heparin to aggregate tetramers of PF4 may also contribute to the ability of PF4/heparin to induce immune cell activation, as aggregated protein antigens are known to be more immunogenic than soluble protein antigens (Wang 2012). Positively charged PF4/heparin complexes may also elicit immune cell activation via their ability to interact with the negatively charged surface of cells (Suvarna*, et al* 2007) since anionic surfaces can enhance the immunogenicity of protein antigens (Yanasarn*, et al* 2011). Our findings are consistent with previous studies demonstrating that positively charged PF4/heparin is immunogenic and suggests that activation of the immune response in HIT may be impacted by the electrostatic charge of PF4/heparin.

Antigenic studies of PF4/heparin demonstrate that antibody binding is highest at a PHR = 1:1, as this PHR produces charge-neutralized complexes that are larger than positively charged PF4/heparin complexes (Rauova*, et al* 2005). Our findings demonstrated that a ten-fold increase in the concentration of heparin (i.e. PHR = 1.3:1) suppresses PF4-dependent proliferation. Consistent with these findings, PF4/heparin complexes with a PHR = 2.6:1 or 1:1 suppresses anti-PF4/heparin antibody generation in mice (Suvarna*, et al* 2007). Furthermore, the ability of PF4/heparin to activate TLR4 is decreased at a PHR = 1.25:1 (Prechel and Walenga 2015), which is consistent with our results demonstrating a dampening of proliferation at an equimolar PHR. PF4/heparin may possess distinct immunogenic and antigenic properties that differently modulate the generation and binding of anti-PF4/heparin antibodies in HIT.

We demonstrate that prior to CPB surgery, no difference exists in the PF4- and PF4/heparin-dependent proliferation response. In addition to exogenous heparin, PF4 also interacts with endogenous GAGs to form PF4/GAG complexes on the surfaces of platelets and monocytes (Rauova*, et al* 2010, Rauova*, et al* 2006). Patients who undergo CPB surgery can suffer from

atherosclerosis (Senay, *et al* 2011), which, in conjunction with aging, are both associated with increased GAG expression (Bilato and Crow 1996). PBMCs derived from these patients may therefore express increased levels of GAGs on their surface, but this has not been demonstrated in the literature. The inability for heparin to enhance the PF4-dependent proliferation response in these patients may be a consequence of the presence of endogenous GAGs, which when in complex with PF4, can induce similar levels of proliferation. PF4 can also form complexes with other endogenous polyanions such as nucleic acids (Jaax, *et al* 2013), but the immunogenic properties of these other PF4/polyanion complexes have not been explored. Our results also demonstrated some variability in the preoperative proliferation response between patients; this could be due to differences in cellular responsiveness to PF4 or variability in other patient-specific variables such as psychological stress, which has been shown to affect mitogen-specific immune cell proliferation (Segerstrom and Miller 2004).

In this patient cohort, our results show that 14 out of 44 (32%) patients produced anti-PF4/heparin antibodies in following CPB surgery. Studies into HIT antibody production have shown that 27%–65% of CPB surgery patients test positive for anti-PF4/heparin antibodies in the postoperative period (Bauer, *et al* 1997, Francis, *et al* 2003, Gluckman, *et al* 2009, Pouplard, *et al* 2005, Trossaert, *et al* 1998, Visentin, *et al* 1996, Warkentin, *et al* 2000, Warkentin, *et al* 2013). In this study, Low molecular weight heparin (LMWH) was used almost exclusively for postoperative thromboprophylaxis and none of the patients in this cohort were administered postoperative heparin without LMWH. LMWH is less immunogenic than heparin (Greinacher, *et al* 2008) and is also less likely to cause HIT (Gruel, *et al* 2003). As of late, various healthcare centres have begun to prescribe LMWH more than heparin for thromboprophylaxis and this shift has been associated with a lower burden of HIT in hospitalized patients (Al-Eidan 2015, McGowan, *et al*

2016). One study that compared antibody production in cardiac surgery patients receiving either heparin or LMWH showed that 29% and 22% of patients tested positive for postoperative anti-PF4/heparin antibodies, respectively (Pouplard, *et al* 1999). In another study, the frequency of postoperative anti-PF4/heparin antibody production was higher for cardiac surgery patients who received heparin compared to LMWH (Visentin, *et al* 1996). The low frequency of anti-PF4/heparin antibody production in this study is consistent with the previously published lower estimates of antibody production in CPB patients, but may also be the result of exclusive postoperative thromboprophylaxis with LMWH. Another factor that may have contributed to the low rate of postoperative anti-PF4/heparin antibody production is that 28 out of 44 (64%) samples were collected early in the postoperative interval and anti-PF4/heparin antibody production is more likely to occur later (i.e. >5–6 days) from the start of heparin therapy (Warkentin, *et al* 2009). Our findings also demonstrated that the highest proportion of patients produced anti-PF4/heparin IgM antibodies before and after CPB surgery. In a study of orthopedic surgery, postoperative LMWH is associated with greater rates of anti-PF4/heparin IgM and IgA antibodies production compared to the IgG isotype (Walenga, *et al* 2004); however, this has not been noted in CPB patients receiving postoperative LMWH. The clinical importance of anti-PF4/heparin IgM antibodies is not well-known, but studies have demonstrated that their presence preoperatively is associated with higher rates of postoperative complications and longer hospital stays (Selleng, *et al* 2010b).

Multiple hypotheses have been posited to explain the ability for PF4 to become immunogenic in a subset of patients administered heparin. One hypothesis suggests that the PF4/heparin-specific immune response is "primed" by exposure to PF4/polyanion complexes prior to heparin exposure (Krauel, *et al* 2011). Atherosclerosis and heart disease are associated with elevated concentrations of PF4 (Levine, *et al* 1981, Pitsilos, *et al* 2003, Sachais, *et al* 2007). We

hypothesized that a subset of these patients have a PF4-responsive immune cell repertoire before surgery that may predispose them to forming anti-PF4/heparin antibodies after CPB surgery. Our findings demonstrate that an association does not exist between preoperative PF4-dependent proliferation and postoperative anti-PF4/heparin antibody production. This analysis, however, is based on a patient cohort with a low rate of postoperative anti-PF4/heparin antibody production and so these findings may not apply to surgical populations with higher rates of postoperative anti-PF4/heparin antibody seroconversion. Proliferation of antigen-specific lymphocyte clones precedes differentiation into effector cells and is required to produce an adaptive immune response (Quah*, et al* 2007); however, we evaluated PF4-dependent proliferation of PBMCs, which contain both innate and adaptive immune cells. As mentioned previously, expression of CD14 seems to increase following *in vitro* stimulation with PF4, a finding that suggests that PF4 is triggering the activation of innate immune cells, which, of course, do not contribute directly to antibody production. Therefore, PF4-dependent PBMC proliferation in this patient cohort may reflect an innate mechanism. Our findings also demonstrated a degree of variability in the preoperative PF4-dependent proliferation response; it is possible that this variability in proliferation is the result of polymorphisms in innate immune receptors such as TLR4. A role for innate immune cells in the PF4-dependent proliferation response provides an explanation for the lack of a relationship between proliferation and antibody production. This study is the first known attempt at utilizing preoperative immunological assays to predict HIT susceptibility in a cohort of surgical patients. One previous study found that the severity of atherosclerotic plaques was not associated with the production of anti-PF4/heparin antibodies after CPB surgery (Cuker*, et al* 2014). These authors concluded that exposure polyanion-modified PF4 in patients with atherosclerotic plaques may not contribute to the postoperative production of anti-PF4/heparin antibodies. Future studies that

utilize immunological markers directly involved in B cell activation may be have a better chance at establishing a relationship with anti-PF4/heparin antibody production in heparin-exposed patients.

HIT is characterized by atypical immunological features such as the rapid generation of anti-PF4/heparin IgG antibodies after primary heparin exposure (Greinacher*, et al* 2009) and the lack of immune memory with repeat heparin exposure (Warkentin and Sheppard 2014). As a result of these unique features, researchers have suggested that innate immune components such as innate-like B cells may contribute to the HIT immune response (Krauel*, et al* 2016, Zheng*, et al* 2013). Qualitative analysis of individual patients demonstrates that two patients who had the strongest PF4-dependent proliferation response before surgery also produced the highest levels of anti-PF4/heparin IgM antibodies after surgery. Anti-PF4/heparin IgM antibodies are unable to cause HIT, but have been observed to rise alongside IgG antibodies in patients diagnosed with HIT (Warkentin*, et al* 2009). Marginal zone B cells and B1 cells are known to secrete IgM antibodies and have both been implicated in the production of anti-PF4/heparin antibodies (Krauel*, et al* 2016, Zheng*, et al* 2013). In humans, innate-like B cells exist within the peripheral circulation of adult humans (Montecino-Rodriguez and Dorshkind 2012, Weller*, et al* 2004); these cells also express innate immune receptors and may become activated following stimulation with PF4 (Prechel and Walenga 2013, Prechel and Walenga 2015). It is possible that innate-like B cells may be involved in the observed preoperative proliferation response and postoperative IgM response in these two patients, but further investigations are required to confirm this notion. Moreover, the preoperative proliferation assay utilized in this study would likely not possess clinical relevance in identifying surgical patients at risk for developing HIT as preoperative proliferation was not predictive with postoperative IgG antibody production in this patient cohort. We cannot, however, conclusively

state that preoperative PF4-dependent proliferation is unable to predict HIT because no patients within this cohort developed HIT postoperatively.

Severe tissue trauma is an independent predictor of HIT (Lubenow*, et al* 2010) and exposure to heparin in a non-inflammatory context can elicit anti-PF4/heparin antibody production (Kelton*, et al* 2012); therefore, we investigated whether a history of major surgical trauma or heparin exposure can predict the magnitude of the preoperative PF4-dependent proliferation response. We were unable to demonstrate that a history of exposure to heparin or surgically induced inflammation can predict preoperative PF4-dependent proliferation. These results are consistent with a potential role for non-anamnestic innate immune cells and not lymphocytes in responding to *in vitro* stimulation with PF4. If PF4 is inducing proliferation of innate immune cell subset(s), we would expect the magnitude of the response to remain consistent across multiple exposures to the same antigen. Again, however, this conclusion is limited by the lack of patients who possessed a high magnitude PF4-dependent proliferation preoperatively. Up to now, HIT studies have not yet analyzed how the pre-heparin immune cell repertoire responds to PF4 and how this may relate to the likelihood of development HIT after heparin exposure.

To further characterize the preoperative PF4-specific immune response, we utilized a PBMC ELISPOT assay to determine whether the preoperative frequency of PF4-specific ASCs can predict postoperative anti-PF4/heparin antibody production. Firm conclusions regarding the association (or lack thereof) between these variables cannot be made for this preliminary data set due to the small sample size (Goodwin and Leech 2006). Our preliminary findings demonstrate that the frequency of preoperative PF4-specific IgG- or IgM-specific ASCs does not predict the postoperative production of anti-PF4/heparin IgG or IgM antibodies, respectively. Similar to the patient cohort used to study PF4-dependent proliferation, these five patients did not produce high

levels of anti-PF4/heparin IgM or IgG antibodies in the postoperative period. CPB surgery has been shown to spontaneously increase the number of postoperative IgG, IgM, and IgA-secreting ASCs specific for various pathogens (Salo*, et al* 1997). Moreover, IgG-specific anti-tetanus toxin and anti-diphtheria toxin antibodies have been shown to increase constantly over a four-month period after CPB surgery (Potschke*, et al* 2012). Other studies have also shown that anti-tetanus toxin antibodies increase by the fifth day after cardiac surgery (Lante*, et al* 2005) and that CPB surgery mediates a shift towards Th2-dominant immune responses (Franke*, et al* 2006). Studies have also demonstrated that lymphocyte repertoires vary before surgery and these authors suggest that this may result from differences in psychological stress levels and medications between patients (Bartal*, et al* 2010). Currently, however, no studies in HIT or other immune-mediated iatrogenic disorders have evaluated the relationship between preoperative B cell frequency and postoperative antibody levels. Recurrent episodes of HIT are unlikely in patients who have had HIT and who are re-exposed to heparin (Potschke 2000) and heparin re-exposure in these patients does not lead to a classic secondary anti-PF4/heparin antibody response (Warkentin and Sheppard 2014). CPB surgery patients who produce postoperative anti-PF4/heparin IgG antibodies have been shown to possess low levels of PF4/heparin-specific memory B cells compared to tetanus toxoid-specific memory B cells (Selleng*, et al* 2010a). In the five patients tested, we have shown a low frequency of preoperative PF4-specific (or PF4/heparin-specific) ASCs, which suggests that exposure to PF4 modified by polyanions prior to heparin cannot generate memory B cells. Consistent with studies demonstrating a lack of anamnesis in HIT, the inability of preoperative PF4-specific ASC frequency to predict postoperative anti-PF4/heparin antibody production might suggest that the immune response in HIT is mediated by innate-like B cells, since these cells do not typically generate memory responses following antigen re-exposure (Defrance*, et al* 2011).

However, further conclusions cannot be made regarding the effect of preoperative B cell frequency on postoperative antibody production given the small sample size, low rate of postoperative anti-PF4/heparin antibody production, and absence of patients who developed HIT.

CPB is an invasive surgical intervention that requires blood to be rerouted through a mechanical apparatus for oxygenation before re-entering circulation. As blood passes through the extracorporeal tubing, it undergoes high rates of mechanical shear and changes in the morphology and function of various blood cells (Rinder*, et al* 1997, Weerasinghe and Taylor 1998). CPB also causes a systemic inflammatory response that can induce widespread cytokine release and activate both innate and adaptive immune cells (Whitlock*, et al* 2015). Our results demonstrate that PF4-dependent proliferation is higher in patients 5–6 days after CPB surgery compared to before surgery. In contrast, unstimulated PBMCs did not demonstrate an increase in proliferation after surgery when compared to before surgery. However, future studies utilizing a non-PF4 protein and a sample of patients undergoing non-CPB cardiac surgery are required to determine whether the observed increase in proliferation is specific to PF4 and CPB patients. Previous studies have shown that CPB increases the proliferation of unstimulated lymphocytes, but not lymphocytes stimulated by mitogens (Salo*, et al* 1997). Cellular exhaustion or the ability for mitogens to maximize proliferation responses may explain the inability for the postoperative lymphocytes to undergo proliferation. PF4/heparin can engage TLR4 and elicit immune activation via cytokine synthesis (Prechel and Walenga 2015). High perioperative concentrations of circulating PF4/polyanion complexes may possess adjuvant-like properties that induce activation of immune cells by interacting with innate immune receptors. Furthermore, CPB is also associated with an increase in TLR4 on human monocytes following surgery (Dybdahl 2001), and CPB leads to the increased production of proinflammatory cytokines one week after surgery (Gasz*, et al* 2006). The observed

increase in postoperative PF4-dependent proliferation may be due to higher levels of responsiveness to PF4 by PBMCs following surgically induced inflammation. Another hypothesis posited to explain HIT immunobiology suggests that a breakdown of regulatory mechanisms may active autoreactive B cells to produce HIT antibodies (Zheng, *et al* 2014). Extracorporeal circulation in surgery can lead to an inhibition of T cell function (Franke, *et al* 2006) and systemic inflammatory response followed by a compensatory anti-inflammatory response (Duggan, *et al* 2006, Gasz, *et al* 2006). Furthermore, *in vitro* stimulation with PF4 has been shown to suppress the function of $CD4^{+}CD25^{+}$ regulatory T cells (Liu, *et al* 2005) and may contribute to a suppression of immune regulatory mechanisms. It is possible that the observed increase in postoperative PF4-dependent PBMC proliferation is partially due to a surgically induced suppression of regulatory mechanisms.

6.0 STUDY LIMITATIONS

In this study, we utilized a prospective cohort design to identify a preoperative immune marker that could predict postoperative anti-PF4/heparin antibody production in patients undergoing CPB surgery. One limitation of the current study is the collection of a single postoperative blood sample over a four-day postoperative interval, as this method of sample collection does not allow for the monitoring of anti-PF4/heparin antibody production over the entire postoperative period. Of the forty-four patients included in the final analysis, over 60% had postoperative samples collected on day 4 and 5, but studies have demonstrated that the likelihood of anti-PF4/heparin antibody production increases with time from CPB surgery (Warkentin*, et al* 2009). Therefore, some of the recruited patients may not have begun to produce anti-PF4/heparin antibodies until later in the postoperative window. Collection of postoperative samples on later days might allow us to establish a clearer relationship between preoperative proliferation and the presence (rather than the absence) of postoperative anti-PF4/heparin antibodies. Furthermore, all the patients included in this study were administered LMWH for postoperative thromboprophylaxis, which is associated with lower rates of anti-PF4/heparin antibody production and a lower incidence of HIT. Recent studies have demonstrated the cost feasibility and reduction in HIT diagnoses with the avoidance of heparin (McGowan*, et al* 2016). Continued avoidance of heparin usage may interfere with future prospective studies attempting to predict the HIT immune response in a cohort of at-risk surgical patients. The chart review protocol utilized in this study was effective for characterizing the heterogeneous patient cohort, but insight into previous surgeries and heparin exposure events was limited by the lack of an integrative electronic charting system that spans healthcare networks outside of Hamilton.

To detect PF4-dependent proliferation we utilized a ^{3}H-thymidine uptake assay, which has dominated studies of cellular proliferation over the previous five decades (Sidman*, et al* 1959). This assay, however, has disadvantages including the usage of radioactive isotopes and the fact that ^{3}H-thymidine is incorporated into the nuclear DNA during the S-phase of the cell cycle, which can be initiated independently of cellular mitosis (Duque and Rakic 2011). Other disadvantages include the time required to perform the assay, and its inability to quantify the levels of cell death and identify the exact cell types that are incorporating the ^{3}H-thymidine (Mead and Lefebvre 2014). As mentioned previously, this proliferation assay is also sensitive to psychological stressors (Segerstrom and Miller 2004), a variable that may have affected patients recruited into this study. Utilization of a heterogeneous pool of PBMCs to detect PF4-dependent proliferation also prevented us from identifying the cellular subset(s) responding to stimulation with PF4.

7.0 CONCLUSIONS

In a subset of individuals, PBMCs are able to undergo proliferation following *in vitro* stimulation with PF4 and this response can be modified by the addition of heparin. These results are consistent with studies into the immunobiology of HIT, which suggest that net cationic PF4/heparin complexes are able to elicit an immune response. We also demonstrated that in a cohort of patients undergoing CPB surgery, preoperative PF4-dependent proliferation is not predictive of postoperative anti-PF4/heparin antibody production. These results suggest that PF4 may not affect the function of lymphocytes and may instead cause the proliferation of innate immune cells prior to surgery. Preliminary evidence also suggests that the preoperative frequency of PF4-specific B cells does not predict postoperative anti-PF4/heparin antibody production, but conclusive statements cannot be made until the sample size is increased. If this trend continues with a larger sample size, it may suggest that exposure to PF4/polyanion complexes may not be involved in the HIT immune response after heparin exposure or innate-like B cells play a significant role in the postoperative production of HIT antibodies. Lastly, we demonstrated that the PF4-dependent proliferation is increased after CPB surgery compared to before surgery, which suggests that the immune system is responsive to PF4 in the immediate postoperative period and this may contribute to the immune response that leads to HIT. Taken together, our findings suggest that the PF4-dependent immune response prior to CPB surgery is unable to predict susceptibility to HIT following heparin exposure, but may provide insight in the PF4-specific immune response following surgery. Future directions may be to assess the ability of markers directly involved in B cell activation prior to surgery to predict HIT antibody production.

8.0 FUTURE DIRECTIONS

To further elucidate the exact cellular subset(s) that proliferate in response to PF4, we would need to utilize EdU or CFSE to stain nuclear DNA in conjunction with various surface markers (i.e. CD19, CD3, and CD14) in order to identify the cell(s) that are proliferating within the pool of PBMCs. Identification of the exact cellular subset(s) that are responding to PF4 will allow us to make conclusions regarding the types and functions of cells that are contributing to the HIT immune response. Furthermore, identification of the responding cell subset(s) will also allow us to perform future experiments to investigate the receptors and cellular pathways involved in the PF4-dependent proliferation response. To investigate the role of GAGs in contributing to PF4-dependent proliferation, future investigations could employ a cell line that lacks the ability to produce GAGs (Schowalter*, et al* 2011), or incubate human PBMCs with an enzyme that cleaves surface GAGs prior to measuring the levels of PF4-dependent proliferation.

The immune response in HIT is atypical and previous studies have implicated murine marginal zone B cells in the generation of anti-PF4/heparin IgG antibodies (Zheng*, et al* 2013). One future investigation could determine whether the number of marginal zone B cells (i.e. $IgM^+IgD^+CD27^+$ cells) in human circulation are increased patients with HIT compared to healthy controls or postoperative CPB patients who do not develop HIT. To further investigate the role for marginal zone B cells in contributing to HIT antibody production, a retrospective cohort design could be utilized to determine whether patients who have had a splenectomy are less likely to develop HIT compared to patients who possess a functional spleen. Another experiment could be to measure whether *in vitro* stimulation of isolated human B cells with PF4/heparin after CPB surgery can induce class-switch recombination as measured by B cell activation markers such as cytidine deaminase. Since the immunobiology of HIT remains an unknown aspect of this disorder,

flow cytometry-based studies of human B cells should investigate whether PF4/heparin is able to crosslink B cell receptors via the detection of CD79b, which contributes to the B cell transduction pathway, and intracellular Ca^{2+}, which is a downstream marker of B cell receptor activation.

The purpose of this study was to utilize preoperative PF4-dependent proliferation to predict postoperative anti-PF4/heparin antibody production. Although this marker was not able to predict postoperative anti-PF4/heparin antibody production, future studies should continue to investigate other preoperative immune markers for their ability to predict patients who may be predisposed to HIT prior to heparin exposure. Identification of a preoperative biomarker for an antibody-mediated disorder may lie in the evaluation of B cell subsets and activation markers prior to surgery. Future studies could assess whether the number of circulating innate-like B cells prior to surgery can predict postoperative anti-PF4/heparin antibody production. Identification of a reliable preoperative marker for HIT in at-risk populations could help prevent the life-threatening consequences associated with this immune-mediated drug reaction.

9.0 FIGURES

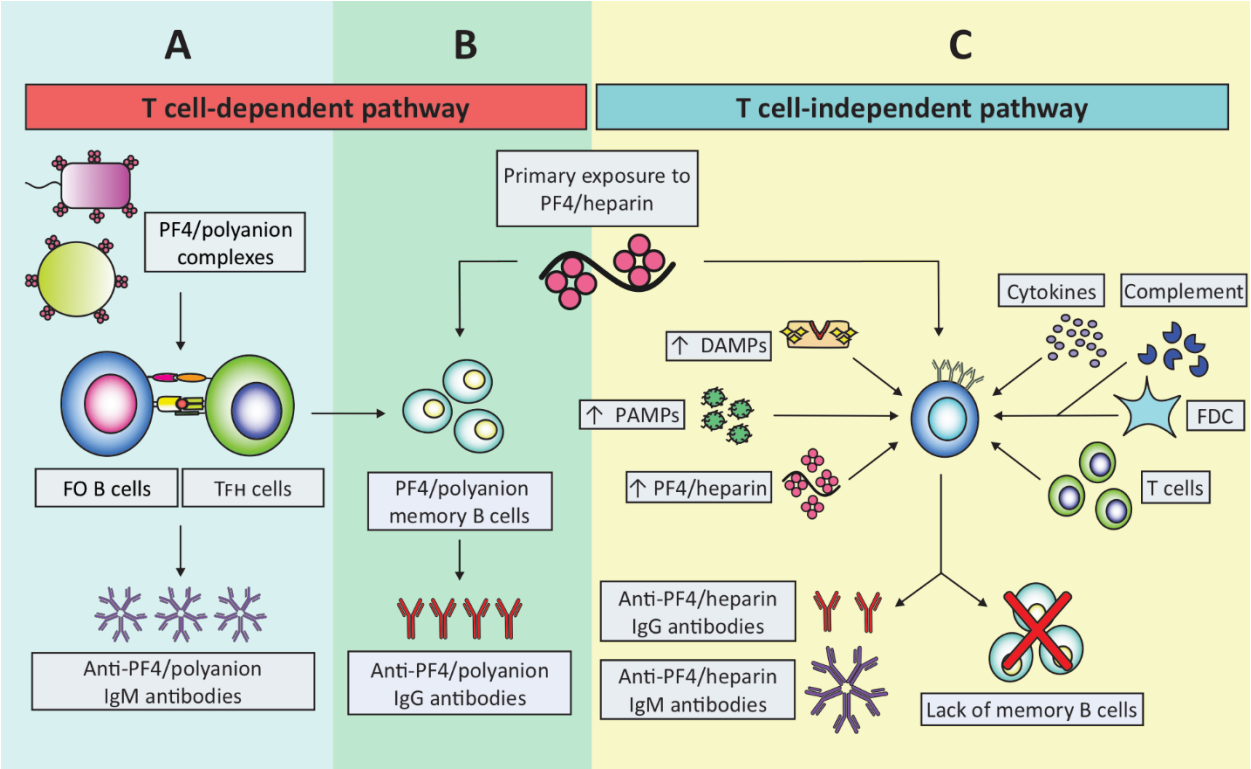

Figure 1. Combined model of HIT immunobiology. Panel A: The HIT-associated T cell-dependent antibody response may begin with primary exposure to PF4/polyanion complexes throughout one's lifetime. These complexes may activate follicular (FO) B cells via follicular T helper (T_{FH}) cells, which interact through major histocompatibility complex (MHC) class II-PF4 peptide interactions to induce anti-PF4/heparin IgM antibody production. Anti-PF4/heparin IgM antibodies may circulate in individuals independent of any heparin exposure. **Panel B:** Another aspect of the T cell-dependent antibody response is the ability for a primary exposure to PF4/polyanion to potentially generate PF4/polyanion-specific memory B cells that can produce anti-PF4/heparin IgG antibodies with primary exposure to PF4/heparin complexes after heparin infusion. These two biological mechanisms may together contribute to the T cell-dependent antibody response in HIT. **Panel C:** The T cell-independent antibody response in HIT may be initiated following primary exposure to PF4/heparin in a surgical setting. PF4/heparin may crosslink B cell receptors (BCR) on B cells. In conjunction with co-stimulation provided by different sources including T cells, danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), high concentrations of PF4/heparin, immunomodulatory cytokines, and/or follicular dendritic cells (FDC) and complement may potentiate rapid production of IgM and IgG anti-PF4/heparin antibodies that contribute to HIT.

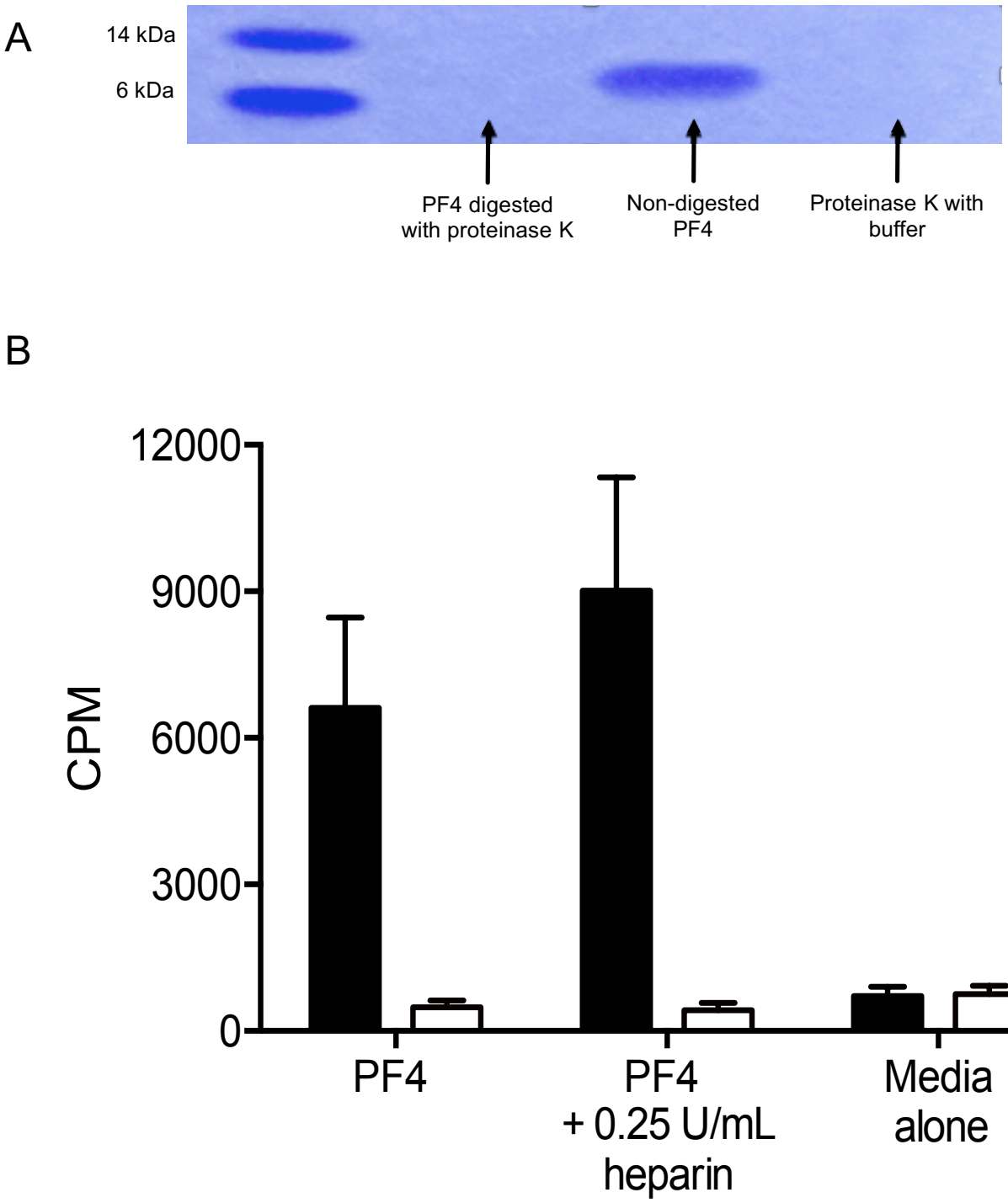

Figure 2. Non-digested PF4 is required to induce PBMC proliferation. A: PF4 (50 μg/mL) digested with proteinase K does not appear on a protein gel, but non-digested PF4 does appear as an 8 kDa band. B: Non-digested PF4 (50 μg/mL) induces proliferation of PBMCs both alone and in complex with 0.25 U/mL heparin (black bars). Digested PF4 does not induce proliferation of PBMCs both alone or in complex with 0.25 U/mL UFH (white bars). PBMCs cultured in media alone do not undergo proliferation in either condition. Error bars represent the mean of six replicate wells ± SEM.

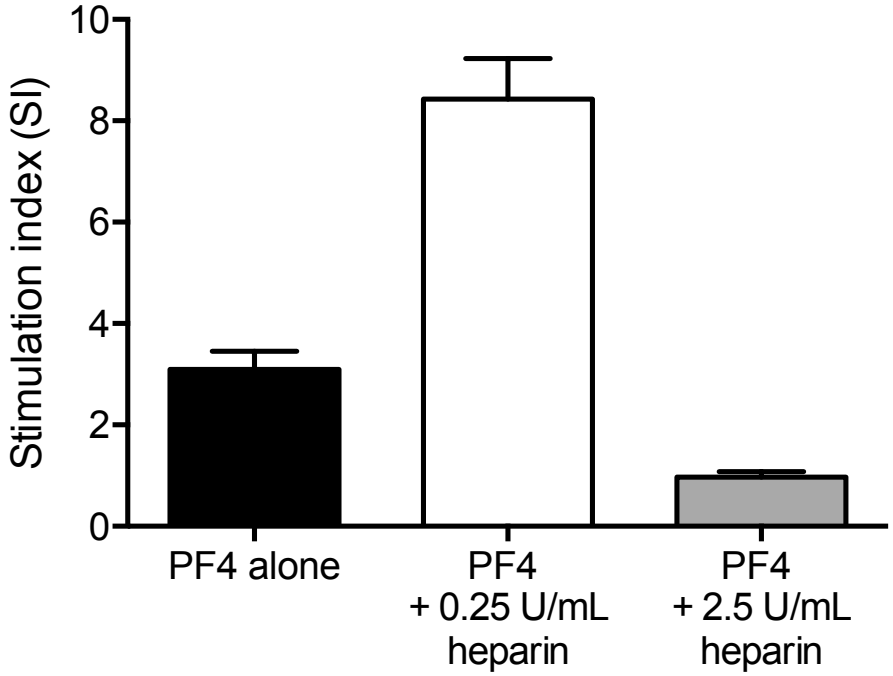

Figure 3. PF4-dependent proliferation is modified by the addition of heparin. In individuals who have PBMCs responsive to PF4 (n=2), proliferation is highest when PBMCs are stimulated by PF4 combined with 0.25 U/mL of heparin. PF4 alone and PF4 combined with 2.5 U/mL of heparin induce lower levels of proliferation. Error bars represent the mean of two individuals ± SEM.

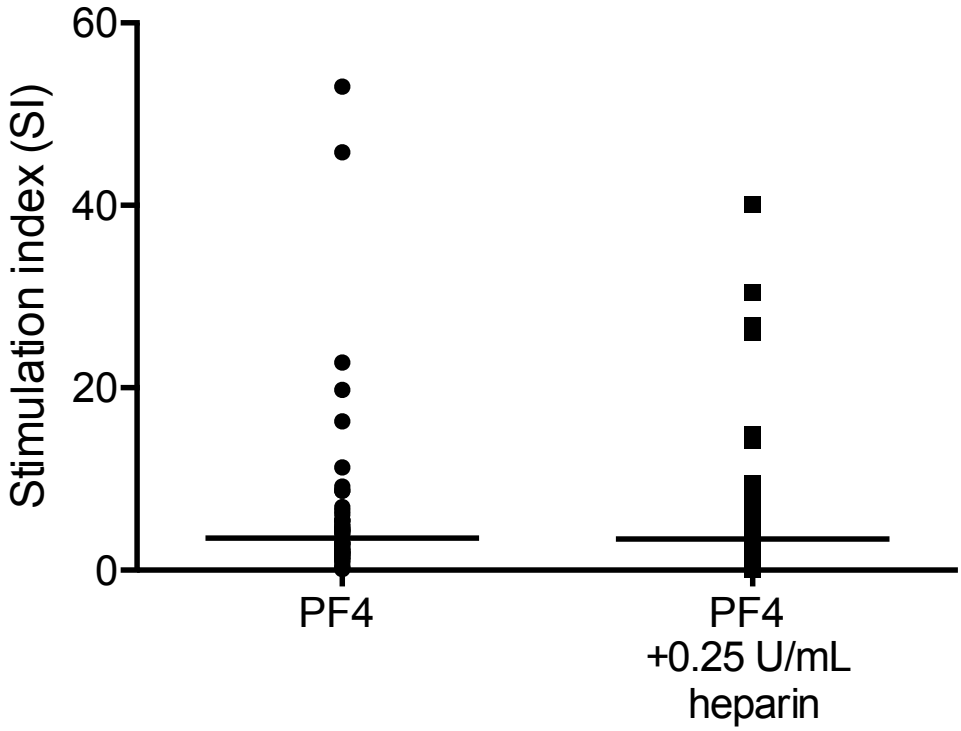

Figure 4. PF4-dependent proliferation and PF4/heparin-dependent proliferation before surgery. No difference exists in the levels of PF4-dependent and PF4/heparin-dependent in this patient cohort before cardiopulmonary bypass surgery [GM PF4 alone proliferation (in SI) vs. GM PF4 combined with 0.25 U/mL heparin proliferation (in SI) ± geometric SEM: 3.54 ± 1.2 vs. 3.43 ± 1.2, $P = 0.93$]. Line represents the geometric mean.

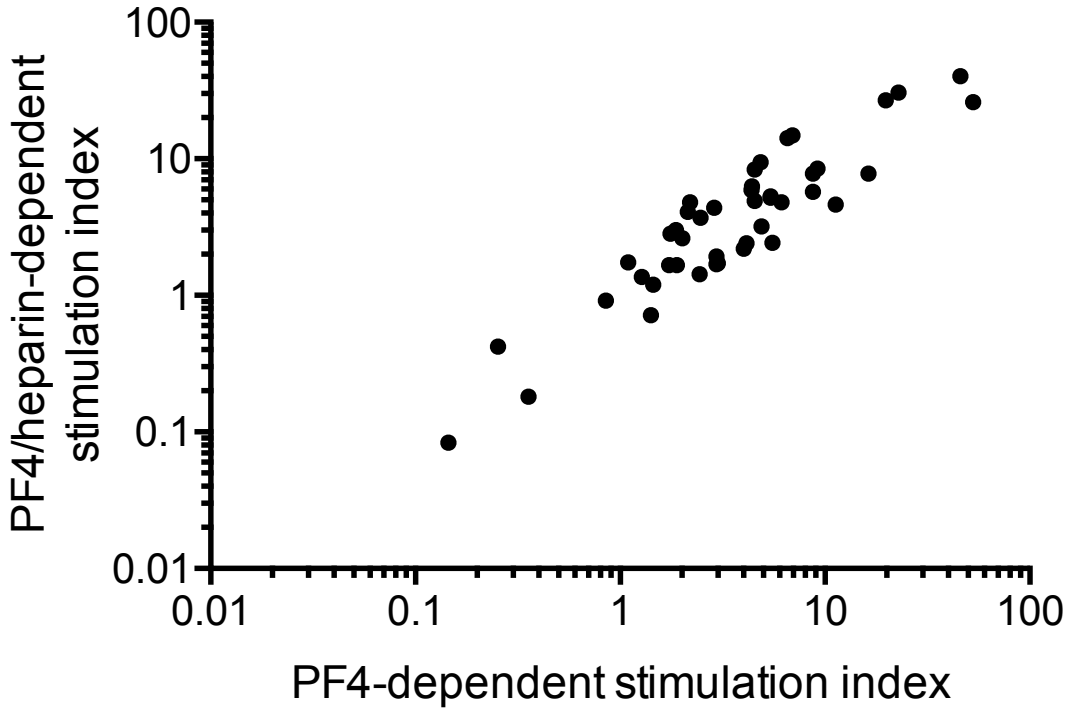

Figure 5. PF4-dependent proliferation is associated with PF4/heparin-dependent proliferation in this patient cohort. Preoperative PF4-dependent and PF4/heparin-dependent proliferation of PBMCs from this preoperative patient cohort demonstrate a strong positive association (Spearman's $\rho = 0.869$, $P < 0.0001$).

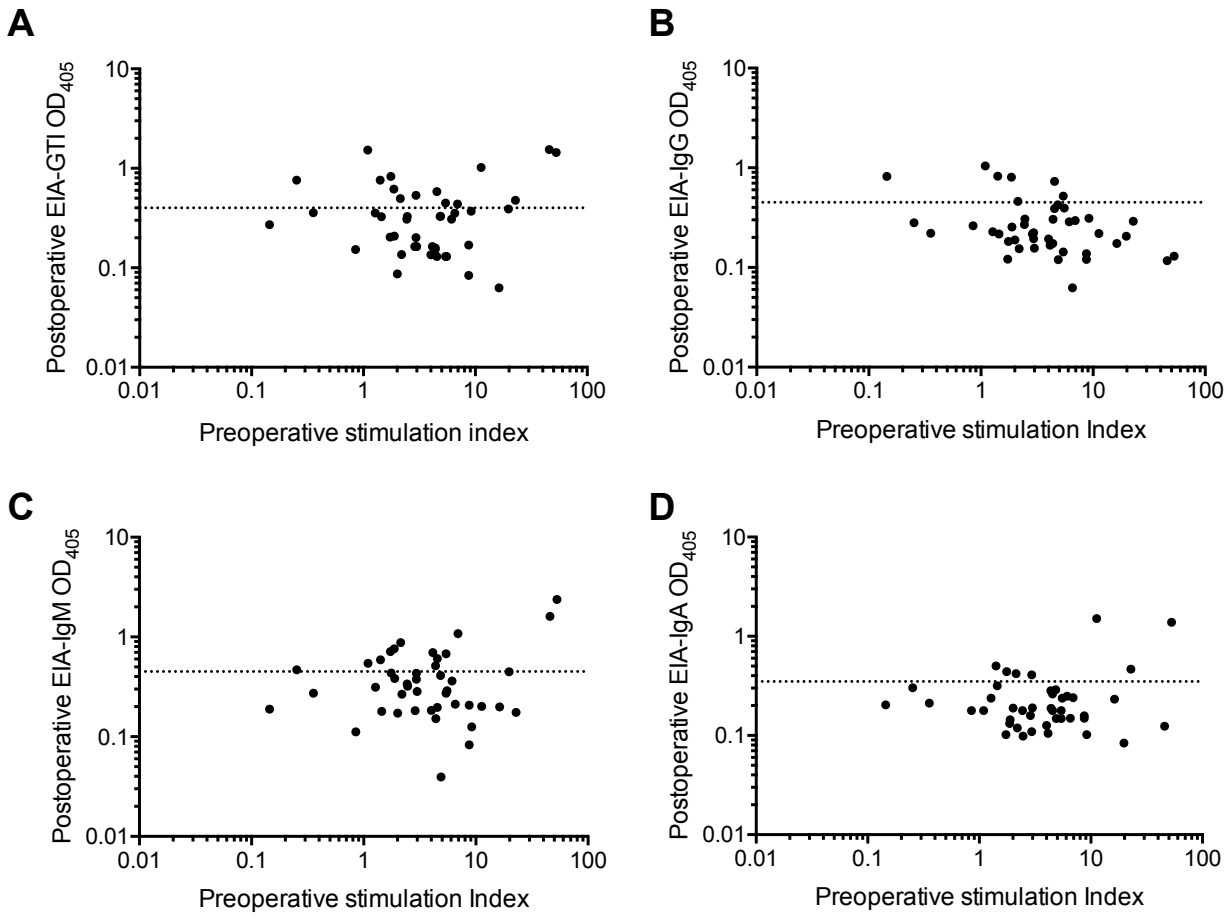

Figure 6A–D. Preoperative PF4-dependent proliferation is not associated with postoperative anti-PF4/heparin antibody production. A: Preoperative PF4-dependent proliferation is not associated with postoperative anti-PF4/heparin antibody levels [Spearman's ρ (95% CI) = –0.02 (–0.32, 0.28), P = 0.91]. Dotted line represents the 0.40 OD_{405} cut-off. B: Preoperative PF4-dependent proliferation exhibits a weak negative association with postoperative anti-PF4/heparin IgG antibody levels [Spearman's ρ (95% CI) = –0.31 (–0.56, –0.02), P = 0.04]. Dotted line represents 0.45 OD_{405} cut-off. C: Preoperative PF4-dependent proliferation is not associated with postoperative anti-PF4/heparin IgM antibody levels [Spearman's ρ (95% CI) = –0.04 (–0.33, 0.26), P = 0.78]. Dotted line represents the 0.45 OD_{405} cut-off. D: Preoperative PF4-dependent proliferation is not associated with postoperative anti-PF4/heparin IgA antibody levels [Spearman's ρ (95% CI) = –0.05 (–0.34, 0.25), P = 0.73]. Dotted line represents 0.35 OD_{405} cut-off.

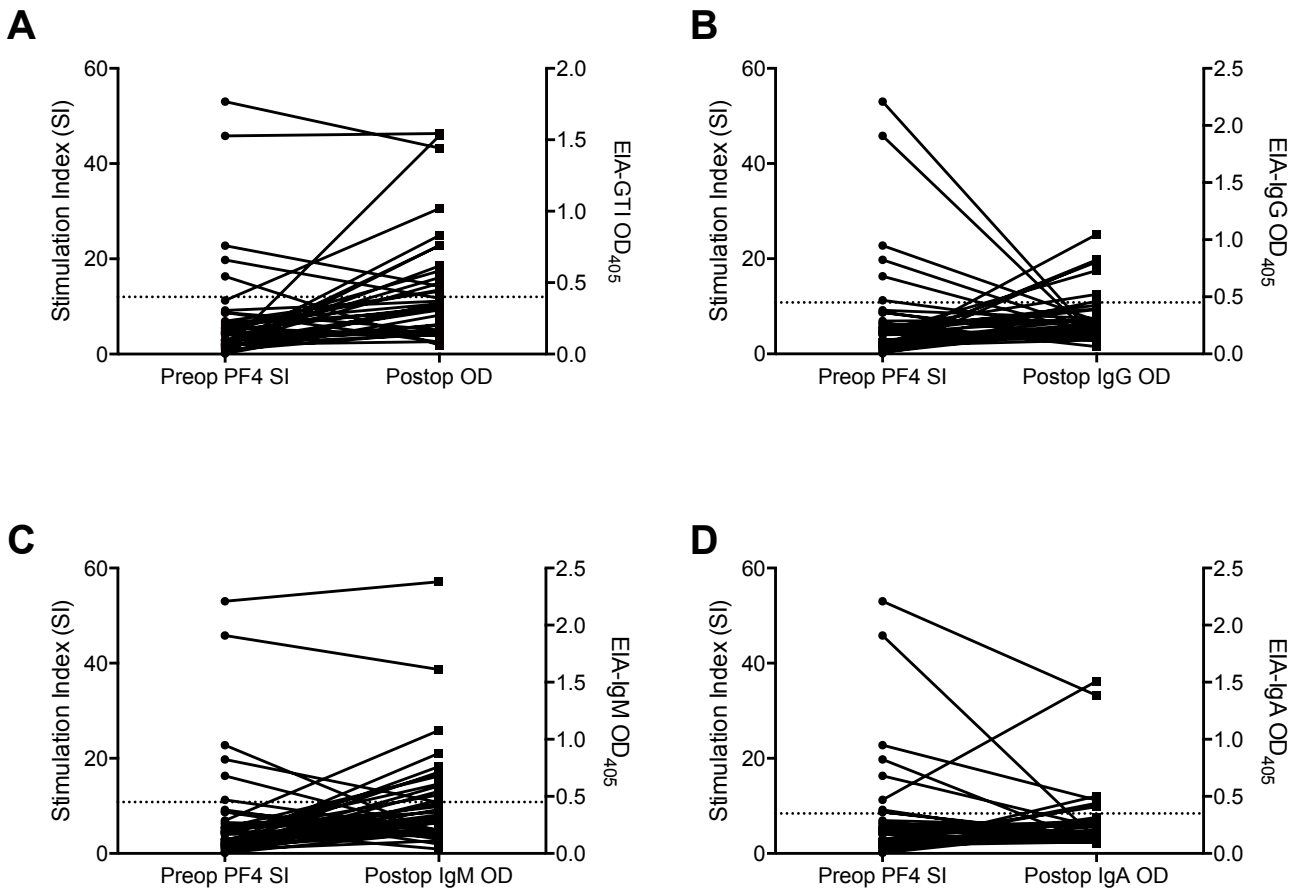

Figure 7A–D. Individual patient analysis of preoperative PF4-dependent proliferation and postoperative anti-PF4/heparin antibody production. A: Polyspecific anti-PF4/heparin antibody levels. Dotted line represents 0.40 OD_{405} cut-off. B: Postoperative anti-PF4/heparin IgG antibody levels. Dotted line represents 0.45 OD_{405} cut-off C: Postoperative anti-PF4/heparin IgM antibody levels. Dotted line represents the 0.45 OD_{405} cut-off. D: Postoperative anti-PF4/heparin IgA antibody levels. Dotted line represents 0.35 OD_{405} cut-off.

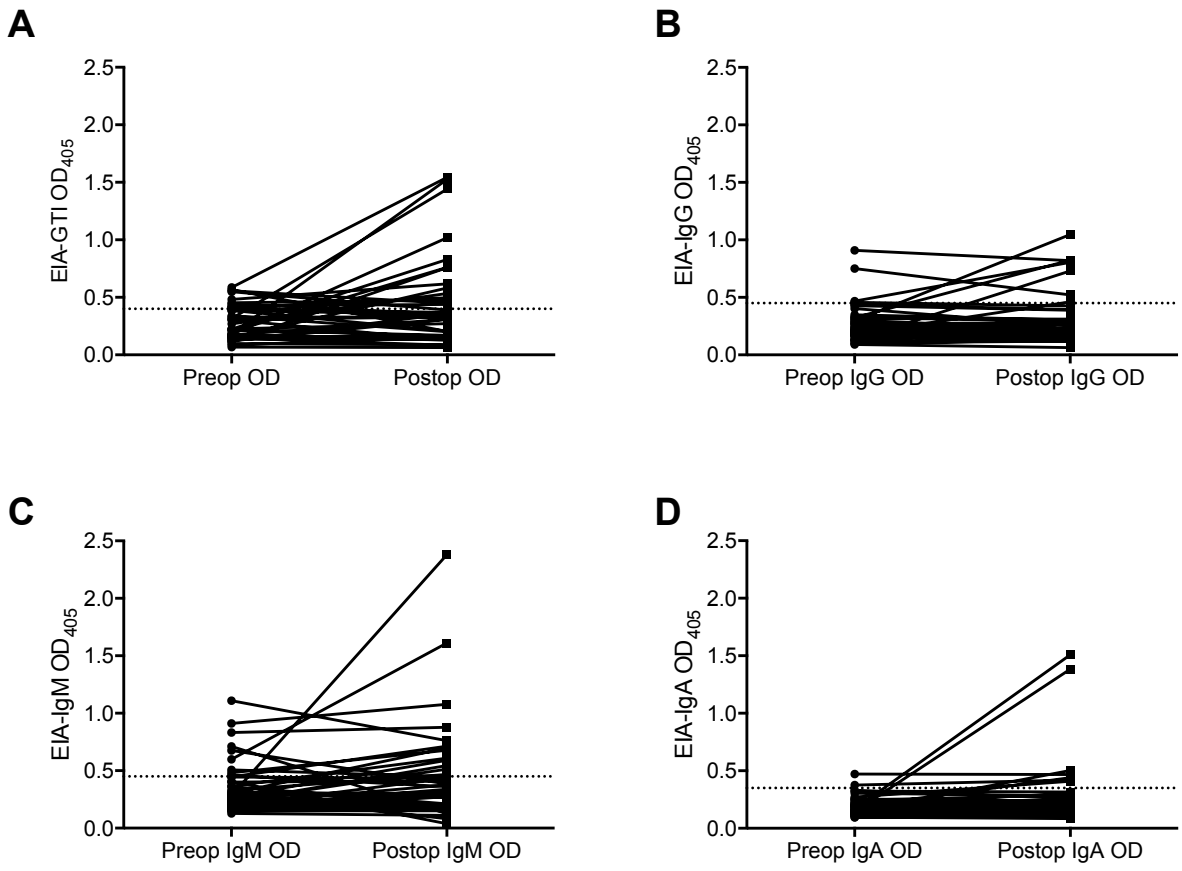

Figure 8A–D. Individual patient analysis of preoperative and postoperative anti-PF4/heparin antibody production. A: Polyspecific anti-PF4/heparin antibody production. Dotted line represents 0.40 OD_{405} cut-off. B: Anti-PF4/heparin IgG antibody production. Dotted line represents 0.45 OD_{405} cut-off C: Anti-PF4/heparin IgM antibody production. Dotted line represents the 0.45 OD_{405} cut-off. D: Anti-PF4/heparin IgA antibody production. Dotted line represents 0.35 OD_{405} cut-off.

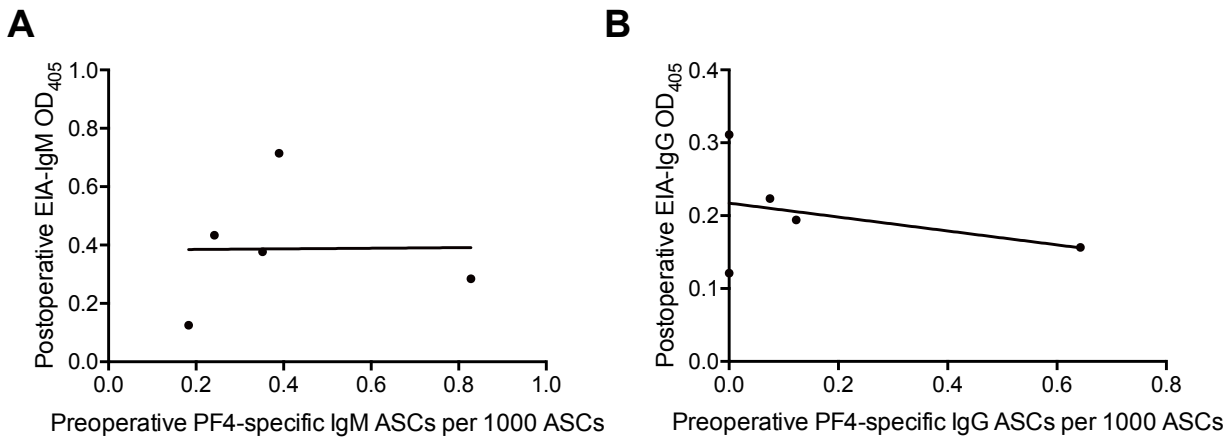

Figure 9A–B. Preoperative frequency of PF4-specific ASCs and postoperative anti-PF4/heparin antibody production. A: Preoperative frequency of PF4-specific IgM ASCs and postoperative anti-PF4/heparin IgM antibody production (Spearman's $\rho = 0.300$, $P = 0.683$). B: Preoperative frequency of PF4-specific IgG ASCs and postoperative anti-PF4/heparin IgG antibody production (Spearman's $\rho = -0.205$, $P = 0.600$).

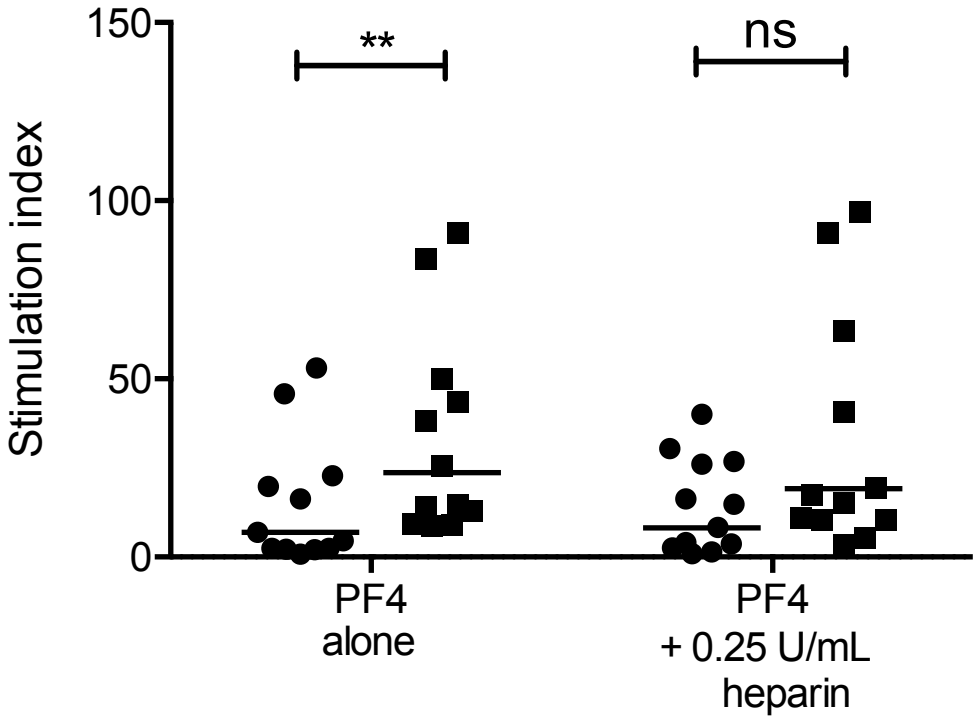

Figure 10. PF4-dependent proliferation increases after cardiopulmonary bypass surgery. *In vitro* stimulation with human platelet-derived PF4 induces higher levels of PBMC proliferation (as measured in SI) after surgery compared to before surgery (23.7 vs. 6.94, $P = 0.009$). *In vitro* stimulation with PF4/heparin also induces higher levels of PBMC proliferation (as measured in SI) post-surgery compared to pre-surgery, but this difference is not significant (19.1 vs. 8.18, $P = 0.064$). PBMC proliferation (as measured in CPM) in media alone does not increase post-surgery compared to pre-surgery (572.5 vs. 624.7, $P = 0.719$) (data not shown). Error bars represent the GM and 95% CI.

10.0 TABLES

Table 1. Baseline demographic and surgical characteristics of patient cohort.

Characteristic	
Baseline characteristics	
No. of patients	44
Age (years)[a]	68.4 ± 10.8
Female	11 (25%)
BMI (kg/m^2)[a]	30.6 ± 8.5
Diabetes mellitus	15 (34.1%)
Preoperative platelet count ($x10^9/L$) [a]	220 ± 57.8
Preoperative leukocyte count ($x10^9/L$)[a]	7.42 ± 1.51
Preoperative hemoglobin concentration (g/L)[a]	140 ± 14.1
Surgery characteristics	
Preoperative cardiac catheterization[†]	42 (95.5%)
Type of surgery	
CABG	24 (54.5%)
Valve surgery	10 (22.7%)
CABG + valve surgery	10 (22.7%)
Intraoperative heparin dose (units)[a,††]	$5.19x10^4 \pm 9.16x10^3$
Cardiopulmonary bypass time (minutes)[a,‡]	100.9 ± 35.8
Cross-clamp time (minutes)[a,‡]	82.7 ± 30
Patients on postoperative LMWH only[‡‡]	42 (95.5%)
Days on postoperative LMWH[a]	6.0 ± 4.5

[a]Mean ± standard deviation (SD). [†]Cardiac catheterization not confirmed for two patients. [††]Heparin dose not confirmed for five patients. [‡]Cardiopulmonary bypass time and cross-clamp time not confirmed for one patient. [‡‡]Two patients underwent additional anticoagulant therapy with either heparin or fondaparinux. BMI, body mass index; CABG, coronary artery bypass grafting; LMWH, low molecular weight heparin.

Table 2. The proportion of cardiopulmonary bypass patients who had positive anti-PF4/heparin antibodies before and 4–8 days after surgery.

PF4-dependent antibody assay	Surgical time-points	
PF4/polyanion-dependent EIA	Pre-surgery[†]	Post-surgery (4-8 days)[††]
Polyspecific	11 (25.0%)	14 (31.8%)
PF4/heparin-dependent EIA		
IgG-specific	4 (9.10%)	7 (15.9%)
IgM-specific	9 (20.5%)	13 (29.5%)
IgA-specific	2 (4.55%)	7 (15.9%)

[†]One patient had both IgM and IgA antibodies and two patients had IgG and IgM antibodies before surgery. [††]Two patients had IgG, IgM, and IgA antibodies, four patients had IgG and IgM antibodies, and one patient had IgM and IgA antibodies after surgery.

Table 3. The levels of anti-PF4/heparin antibodies before and 4–8 days after cardiopulmonary bypass surgery.

PF4-dependent antibody assay	Surgical time-points	
PF4/polyanion-dependent EIA	Pre-surgery[†]	Post-surgery (4-8 days)[††]
Polyspecific[b]	0.24 ± 0.15	0.30 ± 0.37
PF4/heparin-dependent EIA		
IgG-specific[b]	0.23 ± 0.16	0.25 ± 0.22
IgM-specific[b]	0.30 ± 0.22	0.33 ± 0.42
IgA-specific[b]	0.18 ± 0.08	0.21 ± 0.28

[a]Geometric mean ± geometric standard deviation (SD).

Table 4. History of heparin exposure and preoperative PF4-dependent assays.

	Probability of previous heparin exposure			
Baseline	Definite	Possible	Unlikely	*P*
No. of patients	2 (4.55%)	28 (63.6%)	14 (31.8%)	-
Age (years)[a]	76.5 ± 3.54	69.5 ± 9.58	66.1 ± 12.8	-
BMI (kg/m^2)[a]	26.8 ± 3.72	30.2 ± 5.37	31.1 ± 12.4	-
Female	0 (0.00%)	8 (18.2%)	3 (6.82%)	-
Preoperative assay				
Polyspecific anti-PF4/heparin EIA (OD)[b]	0.410 (0.250, 0.673)	0.231 (0.182, 0.294)	0.241 (0.176, 0.331)	*0.387*
IgG-specific anti-PF4/heparin EIA (OD)[b]	0.256 (0.018, 3.61)	0.232 (0.191, 0.281)	0.248 (0.166, 0.234)	*0.852*
IgM-specific anti-PF4/heparin EIA (OD)[b]	0.805 (0.167, 3.87)	0.301 (0.243, 0.372)	0.284 (0.222, 0.364)	*0.091*
PF4-dependent proliferation (SI)[b]	7.98 (1.37, 46.6)	3.98 (2.48, 6.38)	2.81 (1.33, 5.95)	*0.326*

[a]Mean ± standard deviation (SD). [b]Geometric mean (95% CI).

Table 5. The proportion of patients who have had a previous surgical procedure.

Type of surgery	N (%)
Major surgery	
Open-heart surgery*	3 (6.82%)
TKA	4 (9.09%)
THA	1 (2.27%)
Other†	7 (15.9%)
Subtotal	15 (34.1%)
Minor surgery	
Hernia repair	6 (13.6%)
PCI	5 (11.4%)
Other††	10 (22.7%)
Subtotal	21 (47.7%)
No surgery‡	8 (18.2%)
Total	44 (100%)

*Two patients underwent previous CABG or valve surgery. One patient underwent cardiac surgery in childhood. †Includes hysterectomy, cesarean section, spinal surgery, and breast augmentation. ††Includes appendectomy, tonsillectomy, dental extraction, cystoscopy, arthroscopy, and cataract surgery. ‡No surgery noted in medical chart. TKA, total knee arthroplasty; THA, total hip arthroplasty; PCI, percutaneous coronary intervention.

Table 6. History of surgical trauma and preoperative PF4-dependent assays.

	History of surgical intervention			
Baseline	Major	Minor	None	*P*
No. of patients	15 (34.1%)	21 (47.7%)	8 (18.2%)	-
Age (years)[a]	69.6 ± 11.6	69.4 ± 9.69	64.3 ± 12.6	-
BMI (kg/m^2)[a]	30.0 ± 5.99	31.2 ± 10.8	30.8 ± 5.11	-
Female	8 (18.2%)	3 (6.82%)	0 (0.00%)	-
Preoperative assay				
Polyspecific anti-PF4/heparin EIA (OD)[b]	0.226 (0.168, 0.303)	0.230 (0.173, 0.305)	0.283 (0.187,0.428)	*0.653*
IgG-specific anti-PF4/heparin EIA (OD)[b]	0.230 (0.174, 0.305)	0.229 (0.185, 0.283)	0.246 (0.149, 0.406)	*0.924*
IgM-specific anti-PF4/heparin EIA (OD)[b]	0.314 (0.231, 0.427)	0.275 (0.223, 0.340)	0.357 (0.215, 0.593)	*0.609*
PF4-dependent proliferation (SI)[b]	3.50 (1.72, 7.14)	3.80 (2.21, 6.53)	2.99 (1.19, 7.54)	*0.982*

[a]Mean ± standard deviation (SD). [b]Geometric mean (95% CI).

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