

*Curriculum Vitae*

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*Career Profile:* Goal oriented professional with strong analytical skills as demonstrated by scientific background. Possess extensive experience in executing and analyzing laboratory studies. Have strong ability to lead projects and perform exceptionally as a team member. Excellent written and communication skills as demonstrated by previous employment. Interested in pursuing a career providing scientific and technical support in a laboratory environment.

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University of Maryland, Baltimore

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Completed Coursework:

- Methods in Toxicology
- Mechanisms in Biomedical Sciences
- Principles of Pharmacology
- Advanced Toxicology
- Seminar in Toxicology
- Molecular Toxicology
- General Pathology
- Biostatistical Methods
- Readings/Special Topics: Immunotoxicology
- Biomedical Ethics

University of Tampa

Tampa, Florida

*Bachelor of Science, Department of Biology, May 2011*

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Related Coursework:

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- Biological Diversity
- General Chemistry
- Organic Chemistry
- Physics
- Plant Biology
- General Genetics
- General Physiology
- Molecular Biology
- Microbiology
- Cellular Biology
- Immunology
- Behavioral Biology
- Biomedical Ethics
- Psychology
- Biopsychology
- Calculus

*Research Experience*

Graduate Research Assistant

University of Maryland, Baltimore

*December 2013 – Present*

Supervisor: Gary Fiskum, Ph.D.

Primary Research Focus: Investigating the role of mitochondrial bioenergetics in human platelet functionality and storage time using a microplate-based system and western blotting.

Responsibilities: Prepare chemical solutions, media, and reagents. Perform clinical processes involving human blood. Collects and records research data. Assist in conducting research on current scientific literature.

Research Associate

MedImmune, LLC.

*July 2011 – December 2011*

Supervisor: Timothy Pabst, Ph.D.

Responsibilities: Performed purification process development and optimization for monoclonal antibodies and novel protein molecules using ÄKTA™ system; utilized several chromatography modes including: HIC, Ion Exchange, Affinity, and SEC; accomplished column packing and qualification; executed protein concentration and diafiltration by TFF

Oncology Research Intern

MedImmune, LLC.

*Summer 2010*

Supervisor: Keven Huang, Ph.D.

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*Laboratory & Related Skills:*

- Proficient in Microsoft Office and statistical software packages including SigmaPlot and MYSTAT
- Accurate and precise micropipetting
- Reagent preparation
- Handling of biohazardous materials and medical waste
- Separation of whole blood components
- Microscope slide preparation and immunohistochemistry
- Sterile cell culture techniques
- Molecular biology techniques including PCR
- DNA and RNA analysis using gel electrophoresis
- Protein quantification techniques including: Bradford Assay, BCA Assay, ELISA, MSD Assay, Western Blotting techniques
- Protein purification techniques including column chromatography by ÄKTA™ system and using ultrafiltration/diafiltration
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Scientific Program Analyst

NHGRI, National Institutes of Health

*January 2012 – July 2013*

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Public Relations Assistant

University of Tampa

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*Scientific Meetings, Abstracts, & Presentations*

The Cancer Genome Atlas Program Scientific Symposium and Steering Committee Meetings

April 2012, November 2012, May 2013

NHGRI Sequencing Research Network Meeting

National Human Genome Research Institute

October 2012

Annual Meeting of the Return of Results Consortium and Clinical Sequencing

Exploratory Research Program

National Human Genome Research Institute

May 2012, May 2013

## **Abstract**

Extracellular Flux Analysis of Human Platelet Bioenergetics

Lindsey Lund, Master of Science, 2015

Thesis Directed by: Gary Fiskum, Professor, Anesthesiology Department

Availability of platelets for transfusions is limited by a five day maximum storage time which may be caused by a decline in platelet mitochondrial energy metabolism. The metabolic profile of freshly prepared platelets may also reflect acute stress, e.g., that associated with cardiopulmonary bypass surgery, or chronic stress, e.g., that associated with severe depression and fatigue. In preparation for measurements of oxygen consumption by fresh and stored platelets, experiments were performed to optimize platelet isolation and the conditions used during cell respirometry. Preliminary results indicate that maximal platelet respiration declines within 6 days of normal storage and is associated with an increase in apoptosis. No differences in platelet respiration were observed following bypass surgery; however, alterations in metabolic efficiency were observed in two post-stroke patients suffering from depression and fatigue. Platelet respirometry could lead to improved storage times and to its use as a biomarker of bioenergetic dysfunction.

Extracellular Flux Analysis of Human Platelet Bioenergetics

by  
Lindsey Lund

Thesis submitted to the Faculty of the Graduate School of the  
University of Maryland, Baltimore in partial fulfillment  
of the requirements for the degree of  
Master of Science  
2015

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## **Introduction**

### **Summary**

The availability of platelets for massive transfusions is severely limited by the ability of platelets to respond to activation stimuli and initiate the coagulation cascade beyond five days after initial collection. Recent studies have suggested that the limited storage time of platelets is due in part to a decline in mitochondrial energy metabolism. However, rigorous analyses of cellular oxygen consumption by platelets stored for different periods have not been conducted. In preparation for studies, experiments were performed with fresh human blood to optimize isolation of platelets and measurements of platelet bioenergetics.

After establishing appropriate centrifugation parameters and media components, these conditions were applied to platelets isolated from a normal, healthy donor or from cardiopulmonary bypass patients both prior to and following the bypass procedure. The rates of normal, healthy platelet respiration in the presence of different agents that induce maximal and minimal oxygen consumption were consistent over 6 different blood draws from a single donor. As with other cell types, the presence of pyruvate in the medium increased maximal respiration over that obtained with glucose alone, indicating that alternative biofuels can maximize platelet energy production. Under the same conditions, no differences were observed in respiration by platelets obtained immediately prior to and following cardiopulmonary bypass. These results do not support the hypothesis that changes in platelet-dependent blood coagulation after bypass are the result of impaired platelet energy metabolism. Alterations in metabolic efficiency were observed in two post-stroke patients suffering from depression and fatigue.

Preliminary results with platelets stored under standard blood lab conditions indicate that maximal respiration declines by 6 days of storage and is associated with an increase in the Annexin V positive marker of apoptosis. Studies are in progress to test the ability of mitochondria-targeted agents, e.g., pyruvate, to protect platelets against respiratory decline and apoptosis up to 9 days of storage when added to storage media.

### **Importance of Research**

Presently, human blood platelets derived from volunteer donors are administered during massive transfusions for hemorrhagic trauma victims. Platelets collected in the United States by apheresis or within whole blood units undergo processing, pathogen reduction technologies, and may be stored and transfused for up to five days (1). Storage is limited by loss of platelet quality during storage and bacterial contamination. As a result of the short maximum storage life, approximately 30% of stored platelet concentrates are discarded prior to use (1). The limited storage time has major ramifications for United States soldiers stationed overseas. Platelets required for massive transfusions for soldiers stationed overseas must be collected in theatre by apheresis due to the short maximum platelet storage time which prohibits the timely transport and delivery of platelets collected and processed in the continental U.S. Platelets collected at operational theatres in active war zones are not FDA-compliant due to a lack of thorough infectious disease screening prior to or after collection. In addition, some military environments lack the capability to collect apheresis platelets. At these military locations, fresh whole blood units are used as a source of platelets for casualties with life-threatening hemorrhage; this treatment option is also not FDA-approved due to the risk of transfusion-transmitted infections.

The availability of platelets for massive transfusions is severely limited by the ability of platelets to respond to activation stimuli and initiate the coagulation cascade beyond five days after initial collection. Recent studies have suggested that the limited storage time of platelets is due in part to a decline in mitochondrial energy metabolism, oxidative stress, reduced responsiveness to activation, and death of platelets by non-nuclear apoptosis (2). A significant prolongation of platelet storage time would potentially allow for delivery of FDA-approved platelet concentrates from the United States to military operational theatres abroad and reduce the reliance of operational theatres on fresh whole blood transfusions, thereby minimizing the risk of transfusion-transmitted infections.

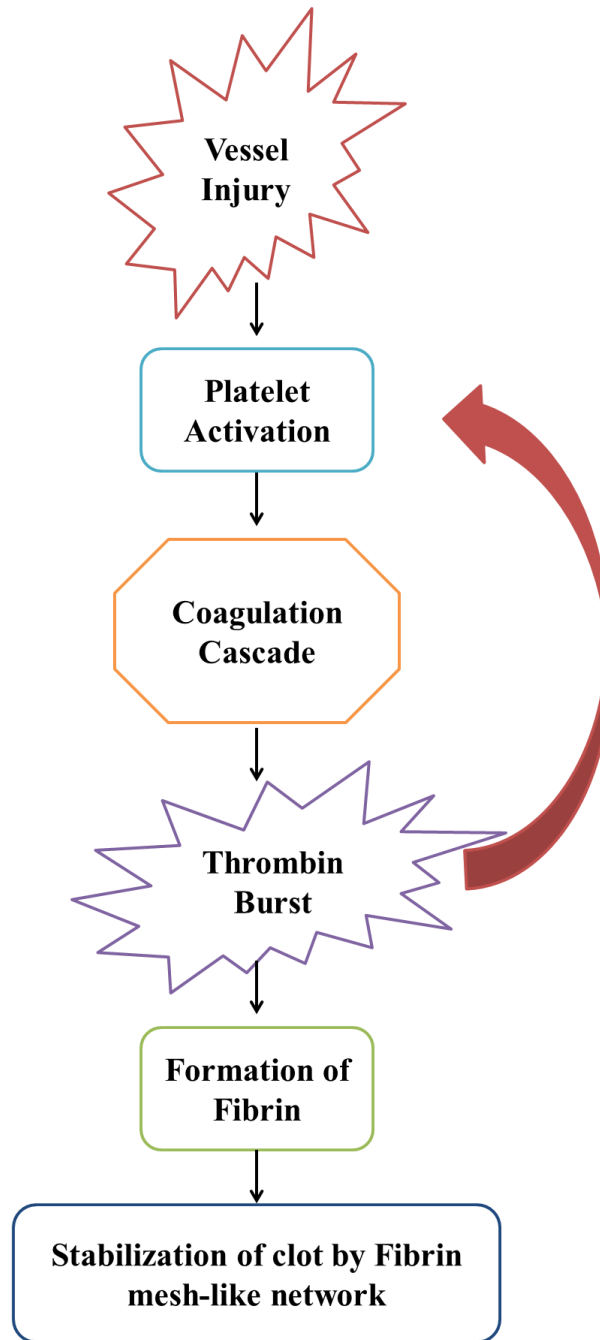
The primary goal of the project is to determine the best storage medium for isolated platelet concentrates to minimize mitochondrial dysfunction, maintain robust response to platelet aggregation agonists, thereby prolonging storage time beyond five days. We hypothesize that platelet storage time is limited by mitochondrial energy metabolism and that the inclusion of alternative biofuels, antioxidants, and other mitochondria-targeted agents attenuates mitochondrial dysfunction, allowing for increased platelet storage time

### **Introduction to Platelet Physiology**

Platelets are small, anucleate particles derived from megakaryocytes that circulate in the blood and play a critical role in blood clot formation to minimize bleeding (3, 4). Recent studies have demonstrated that beyond preventing excessive blood loss, activated platelets also have the ability to initiate signal-dependent protein synthesis, trigger local inflammatory responses, and synthesize lipid mediators (4, 5). Platelets circulate

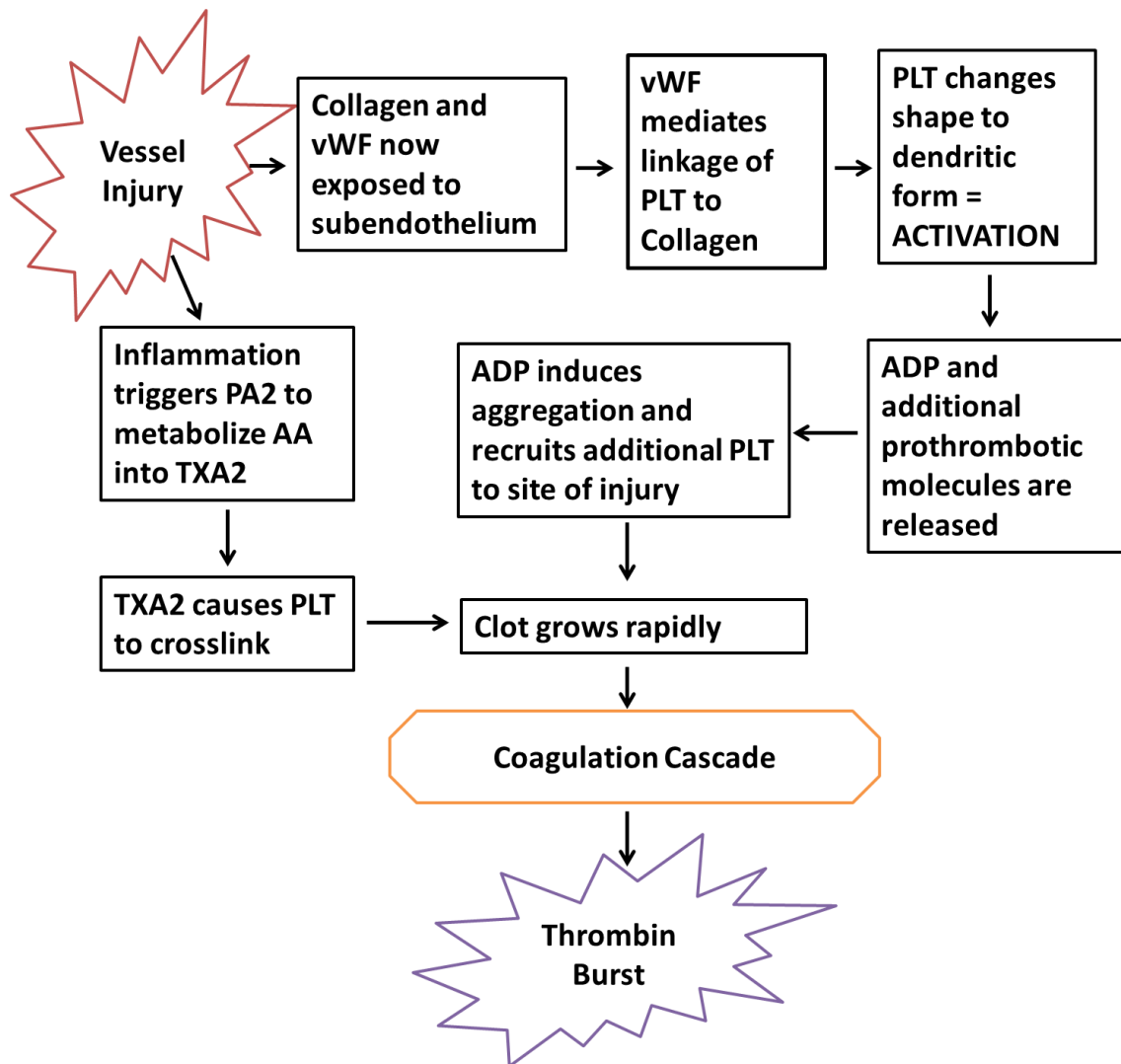
throughout the blood in an inactive state until they encounter endothelial damage or are activated by the coagulation cascade (4, 6). Activation causes the platelets to change shape, release their granule contents, and aggregate together to terminate blood loss (4). Previous studies have demonstrated that metabolic energy is required for platelet responses to stimuli; platelet activation accelerates ATP-regenerating sequences while metabolic inhibitors prevent platelet responses (7).

In response to vascular damage, platelets quickly activate and adhere to the damaged vessel wall at the site of injury, triggering a series of events that recruits additional platelets for thrombus formation, mobilizes leukocytes to combat pathogens that may have entered at the site of injury, and activates the blood coagulation cascade (8, 9). Reference diagrams are included to illustrate the role of vessel injury and the coagulation cascade in platelet activation and functionality (See Figures 1, 2, 3).



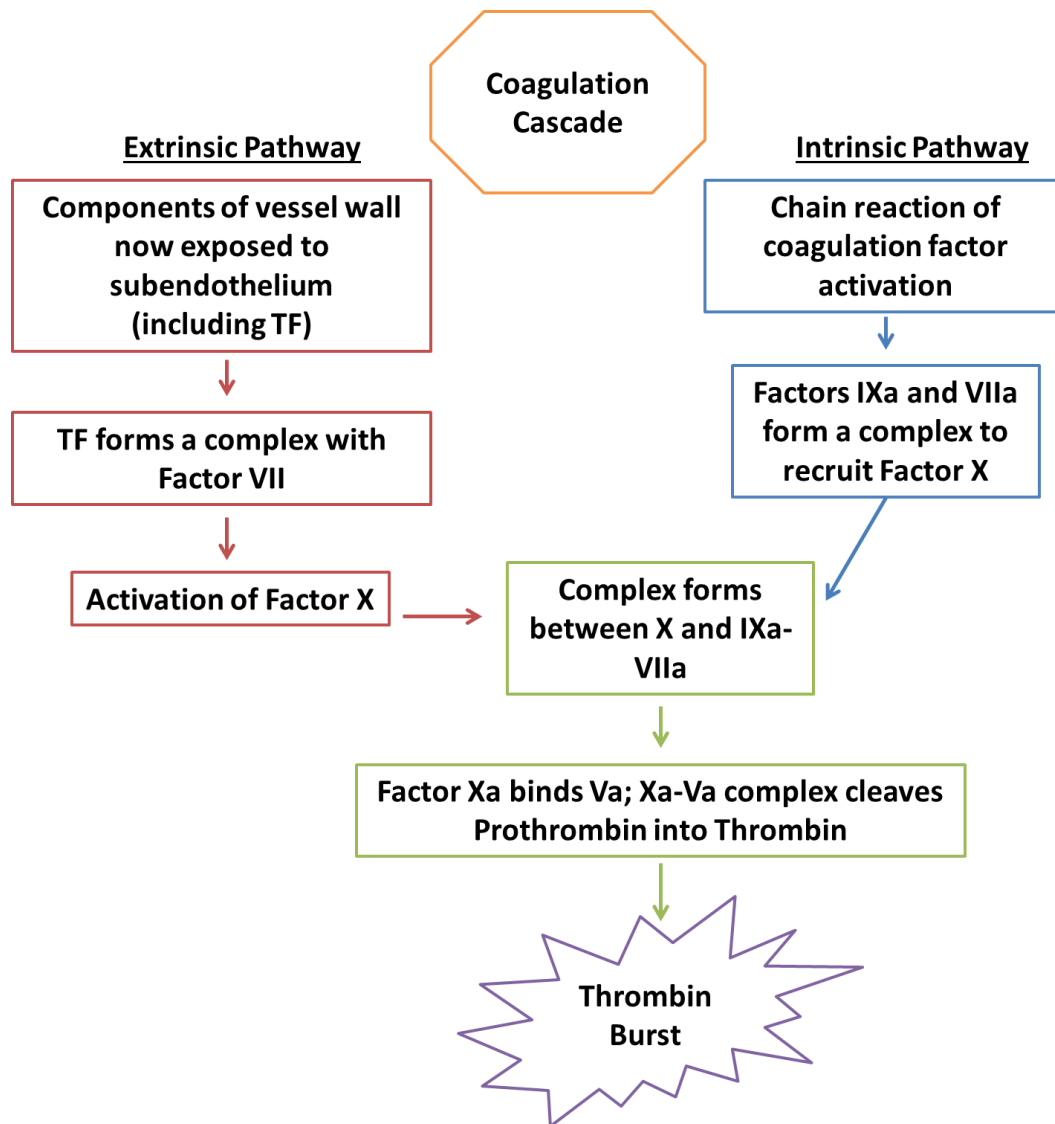
**Figure 1: Platelet Activation Initiated by Vessel Injury**

Vessel Injury induces platelet activation leading to formation of a hemostatic plug in the form of a fibrin mesh-like network.



**Figure 2: Vessel Injury Promotes Release of Prothrombotic Mediators**

Vessel Injury exposes collagen and Von Willebrand Factor (vWF) to the subendothelium. vWF mediates the linkage of platelets (PLT) to collagen resulting in platelet activation and shape change. Additional prothrombotic molecules, including ADP, are released. ADP induces aggregation and recruits additional platelets to the site of injury. Simultaneously, inflammation at the site of injury triggers Phospholipase A2 (PA2) to metabolize Arachadonic Acid (AA) into Thromboxane A2 (TXA2). TXA2 induces platelet crosslinks. The clot at the site of the injury grows rapidly and triggers the coagulation cascade leading to thrombin burst.



**Figure 3: Coagulation Cascade**

Contributions of the intrinsic and extrinsic pathways of the coagulation cascade in initiation of the thrombin burst.

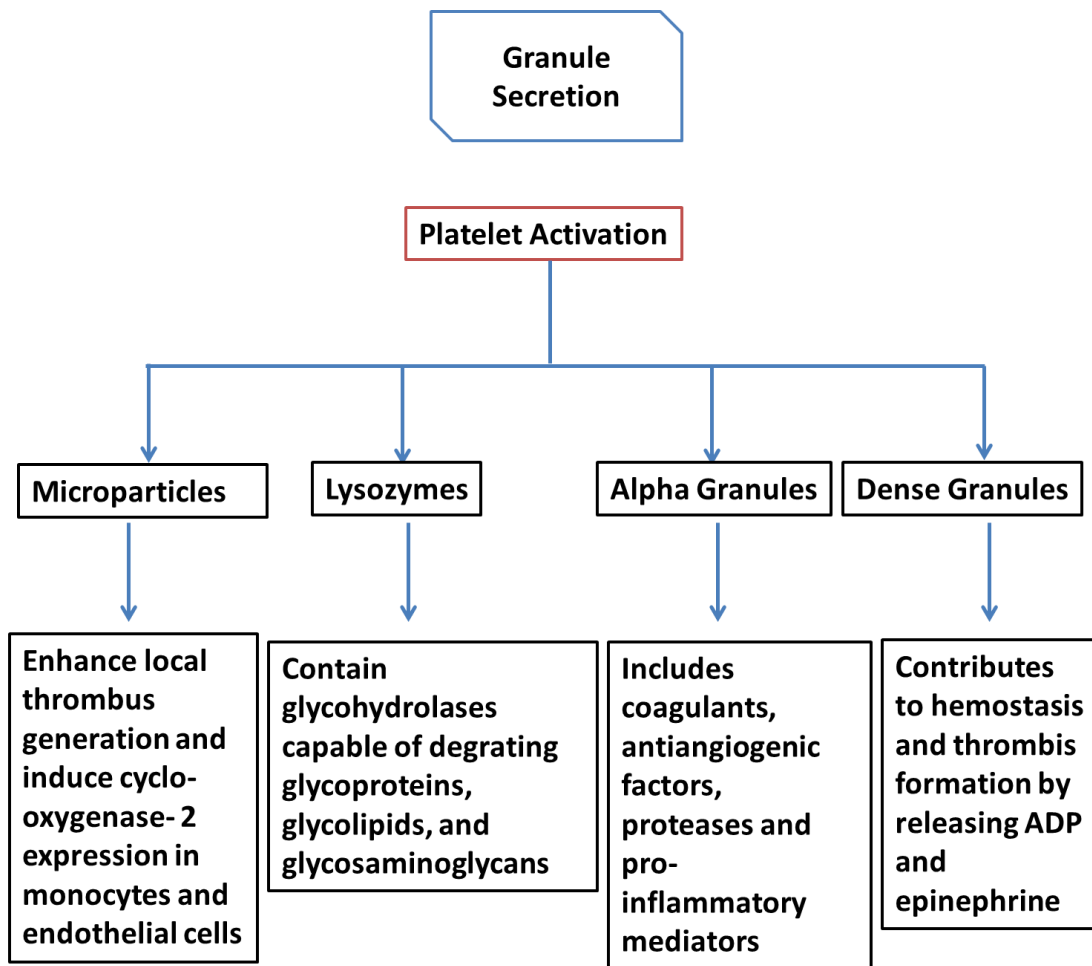
Platelet activation is triggered by the presence of various agonists at the site of endothelial injury (4). Endothelial damage releases multiple proteins normally present in the extracellular matrix into the blood, including collagen and Von Willebrand factor (9). Inflammation at the site of injury triggers phospholipase A<sub>2</sub> to metabolize arachidonic acid into thromboxane A<sub>2</sub>. Presence of thromboxane A<sub>2</sub> and collagen initiates a

coagulation cascade which cleaves prothrombin into its active form, thrombin. Thrombin quickly converts fibrinogen into insoluble strands of fibrin to promote clotting at the site of injury. Finally, ADP and additional soluble factors are released from granules within the platelets to recruit additional platelets and inflammatory mediators to the site of injury (9).

Binding of collagen to receptors on platelets activates a signaling cascade that hydrolyzes phosphatidylinositol bisphosphate (PIP<sub>2</sub>) to inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) and activates PKC (10). IP<sub>3</sub> releases calcium from intracellular stores and DAG promotes calcium entry from extracellular compartments (10). Calcium mobilization from intracellular and extracellular stores contributes to multiple phases of platelet activation, including reorganization of the cytoskeleton, integrin activation, and granule release (10). The accumulation of calcium also magnifies weak stimuli and improves platelet pro-coagulant activity (10).

Granule secretion is accomplished by a highly dynamic cytoskeleton within the platelets that is continuous with the plasma membrane; the open canicular system provides a contractile system involved in shape changes, pseudopod formation, and granule secretion during platelet activation (8, 11). Upon activation, platelets release platelet-derived microparticles, lysozymes, and the contents of their alpha and dense granules (Refer to Figure 4) (4). Membrane microparticles released by platelets enhance local thrombus generation and induce COX2 expression in monocytes and endothelial cells (4). Lysosomes released by activated platelets store glycohydrolases able to degrade glycoproteins, glycolipids, and glycosaminoglycans (12). In addition to degradation of proteins, carbohydrates, lipids, and nucleic acids both in and outside of the cell, recent

studies have discovered a possible contribution of platelet-released glycohydrolases in vascular remodeling following vessel injury (12). Alpha-granules contain hundreds of proteins including coagulants, antiangiogenic factors, proteases, and proinflammatory mediators (13). Dense granules contribute to hemostasis and thrombosis by releasing ADP and epinephrine (13).



**Figure 4: Granule Secretion**

Platelet activation results in granule secretion and release of microparticles and lysozymes.

Degranulation is critical in the regulation of acute hemostasis and thrombus formation and it requires an enormous amount of metabolic energy (7). Verhoeven *et al.* demonstrated that the velocities of granule secretion vary in parallel with the rate of energy consumption in thrombin-stimulated platelets (7). Thrombin stimulation initiated shape change and granule secretion in platelets; these changes were accompanied by an increase in energy consumption of 8  $\mu\text{mol}$  of ATP per minute per  $10^{11}$  platelets (7). The initial increase in energy consumption observed after thrombin addition was reduced by more than 50% when the platelets were preincubated with Hirudin, a direct thrombin inhibitor (7). Hirudin alone did not affect energy consumption, trigger a functional response, or result in shape change (7). Inhibition of oxidative phosphorylation by *in vitro* exposure of platelets to respiratory inhibitors, such as antimycin A, depresses agonist-induced granule secretion and aggregation, thereby reducing platelet functionality (7).

ATP and ADP are released from secretory molecules at approximately equal molar concentrations (14). ADP is known to interact with P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors on platelets to induce platelet aggregation. ATP can interact with the same receptors resulting in transient Ca<sup>2+</sup> mobilization, but this interaction does not contribute to platelet aggregation (14). High concentrations of ATP are known to inhibit platelet activation through antagonism of P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors (14). A recent study by Stafford *et al.*, demonstrated the ability of ATP to induce platelet activation in whole blood but not in platelet rich plasma (PRP). Increasing concentrations of ATP from 10 to 100  $\mu\text{mol/L}$ , added to whole blood samples, stimulated platelet aggregation (14). However, ATP was unable to induce aggregation response in PRP at any concentration (14). The ability of

ATP to induce aggregation in whole blood requires the conversion of ATP to ADP by ecto-ATPases on blood cells including polymorphonuclear leukocytes and mononuclear leukocytes (14). The addition of enzymes capable of dephosphorylating ATP into ADP to PRP rescued the ability for platelets suspended in plasma alone to aggregate (14).

### **Platelets as Biomarkers of Pathologies**

Despite their small size and lack of a nucleus, a few mitochondria are present within each platelet, playing essential roles in activation, maintenance of cellular redox homeostasis, and non-nuclear apoptosis; maintenance of a healthy mitochondria population is critical for platelet functionality (3). Elucidating the mitochondrial bioenergetic profile for healthy basal or activated platelets could provide the framework for understanding the deleterious consequences of prolonged platelet storage. In addition, measurement of platelet bioenergetics may be used to compare platelet vitality in different storage mediums.

Recent advances in measuring cellular bioenergetics in living cell populations have demonstrated that a cell's bioenergetics capabilities can serve as an indication of healthy mitochondrial populations and overall cell viability; cells experiencing mitochondrial dysfunction may succumb to cell death if energy requirements are not satisfied (15). Pathologies resulting in clinical symptoms that affect single or multiple organ systems could have the potential to alter the mitochondrial populations in platelets (16). A recent study published by Cardenes *et al*, analyzed platelet bioenergetics in patients with sickle cell disease; they observed deficient complex V activity leading to decreased mitochondrial respiration and membrane hyperpolarization compared to healthy controls (17). This dysfunction correlated with platelet activation and hemolysis

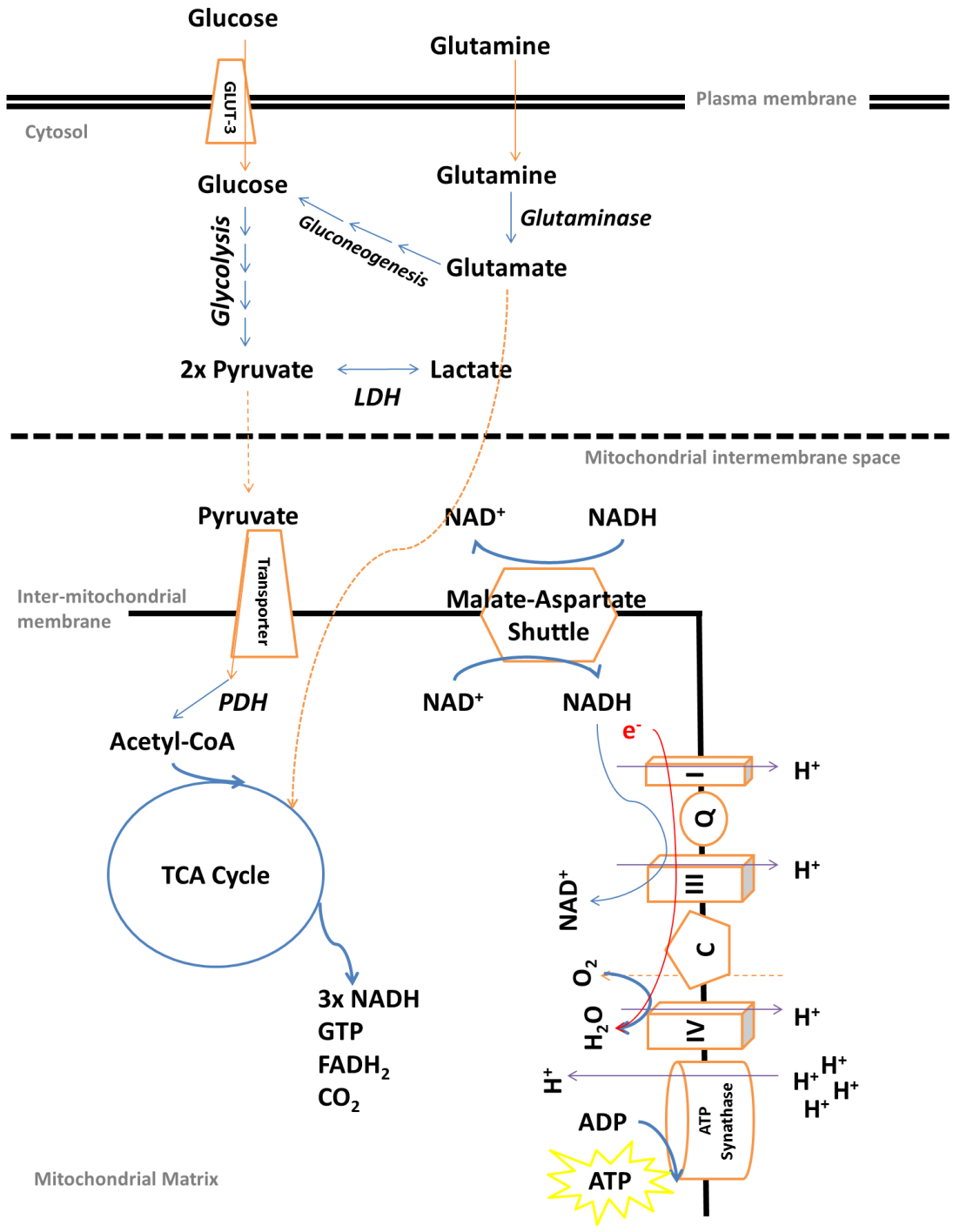
which may potentially be attenuated with the use of mitochondrial-targeted therapeutics (17). In another study, patients with Type II diabetes exhibited perturbations in oxygen consumption and lower oxygen-dependent ATP synthesis compared to healthy controls (18). These preliminary studies have demonstrated that platelets have the potential to be used as biomarkers for pathologies involving aberrant bioenergetics. Elucidating the mitochondrial bioenergetics profile of platelets from healthy controls could help to identify mitochondrial dysfunction in platelets from patients with various pathologies.

Previous studies have demonstrated that cardiopulmonary bypass activates platelets resulting in structural and biochemical changes (19). Alterations in platelet count, clot formation, and platelet interactions with other cells have been observed (19). Physiological factors such as increased shear force, exposure to artificial surfaces, and the use of exogenous drugs could induce changes in platelet function (19). The ramifications of cardiopulmonary bypass on platelet bioenergetics have not yet been evaluated. We hypothesized that changes in platelet-dependent blood coagulation after bypass are the result of impaired platelet energy metabolism.

### **Platelet Metabolism**

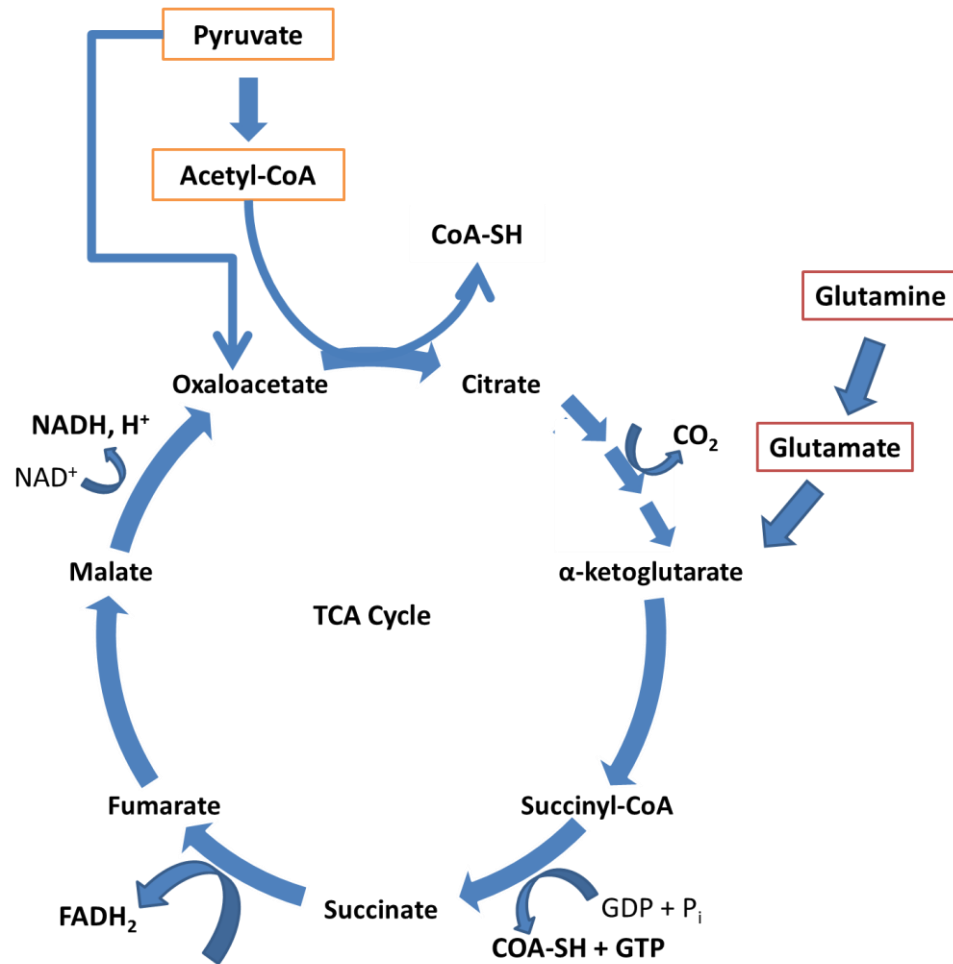
In their basal state, platelets actively uptake glucose from the surrounding environment via the glucose transporter GLUT-3 located in their plasma membrane (2). Like most cells, platelets utilize glucose to generate energy in the form of ATP by anaerobic glycolysis or aerobic oxidative phosphorylation (Refer to Figure 5). In the cytosol, glycolysis metabolizes one glucose molecule into two pyruvate molecules, resulting in the formation of three ATP molecules by substrate-level phosphorylation. In the presence of oxygen, pyruvate is further metabolized within the mitochondrial matrix,

resulting in the generation of an additional approximately 30 ATP molecules; decarboxylation of pyruvate by pyruvate dehydrogenase complex into Acetyl-CoA provides the necessary substrates for the Tricarboxylic Acid (TCA) cycle to generate NADH, FADH<sub>2</sub>, CO<sub>2</sub>, and ATP or GTP. Products of the TCA cycle, including NADH and FADH<sub>2</sub>, are used by the electron transport chain (ETC) located within the innermitochondrial membrane (Refer to Figure 6). During oxidative phosphorylation, electrons are transferred from NADH and FADH<sub>2</sub> to Complex I and II, respectively. The electrons are then shuttled from Complexes I and II to Coenzyme Q, passed to Complex III, and finally ferried to Complex IV. As electrons move through the ETC, Complexes I, III, and IV allow hydrogen ions (protons) in the mitochondrial matrix to move into the intermembrane space. The efflux of protons across the inner membrane establishes the relatively large negative mitochondrial membrane potential. The electron transport chain-dependent efflux of protons across the inner membrane is balanced by the influx of protons specifically through the proton pore component of the ATP Synthase. The flow of hydrogen ions flowing down their electrochemical gradient results in major changes in structural conformation and binding affinities for ADP, inorganic phosphate, and ATP, resulting in the endergonic synthesis of ATP. During oxidative phosphorylation, oxygen serves as the final electron acceptor at Complex IV of the electron transport chain and is consumed to generate water. Supplementary to the production of water and ATP, the series of reactions involved in oxidative phosphorylation also produce intermediates for the biosynthesis of other molecules required for cell survival (2).



**Figure 5: Cellular Respiration**

Cellular respiration is the process by which the chemical energy of substrate molecules is released and partially captured in the form of ATP. Glucose is the most commonly used fuel.



**Figure 6: TCA Cycle**

Inside the mitochondrial matrix, the TCA cycle generates chemical energy (ATP, NADH, and FADH<sub>2</sub>) from the oxidation of pyruvate and α-ketoglutarate.

As mentioned previously, platelets obtain glucose from their environment via the GLUT-3 transporter. If the uptake of glucose into the platelet by GLUT-3 exceeds the ability of it to be aerobically metabolized, pyruvate generated from glucose will be converted into lactate by lactate dehydrogenase (20). Alternatively, platelets may be able to indirectly avoid producing lactate by activating the hexose monophosphate shunt. The hexose monophosphate shunt utilizes glucose to generate biosynthetic intermediates and NADPH when energy production is not required or if a preferred fuel is available (2). In

erythrocytes, approximately 10% of glucose consumption is metabolized by the hexose monophosphate shunt (21). Oxidative stress increases metabolism of glucose by the hexose monophosphate shunt; more than 80% of total glucose consumed by erythrocytes may be directed through this pathway (21). The ability to modify the mechanism of glucose consumption in erythrocytes during periods of stress suggests that platelets may also be able to shift their metabolic activities to increase glucose metabolism by the hexose monophosphate shunt, thereby indirectly reducing the amount of glucose available for metabolism by lactate dehydrogenase. Further studies are necessary to fully describe the role of the hexose monophosphate shunt in platelets.

Under normal physiological conditions, aerobic metabolism by oxidative phosphorylation contributes to 85% of energy production in platelets (2, 22, 23). Platelets maintain high ATP/ADP and NADH/NAD<sup>+</sup> ratios under baseline conditions (2). A study of bioenergetic profiles in blood cells, normalized to  $\mu\text{g}$  protein, found that compared to monocytes and lymphocytes, platelets possess a higher level of ATP-linked oxygen consumption and a lower amount of proton leak (22). Thus, anaerobic energy metabolism in platelets is highly efficient, relative to leukocytes. More than 60% of basal oxygen consumption in platelets is attributed to ATP-linked respiration, twice that of monocytes and lymphocytes (22). While the maximal oxygen consumption rate (OCR) stimulated by an uncoupler was similar for platelets and other blood cells, the reserve capacity is significantly lower in platelets compared to monocytes. The reserve capacity is the difference between basal and maximal OCR. A low reserve capacity indicates a reduced quantity of spare mitochondrial energy to use in stress-inducing conditions (22). Roughly 35% of maximal OCR in monocytes and lymphocytes is ascribed to reserve capacity

while less than 20% is dedicated to reserve capacity in platelets (22). These findings suggest that platelets have high levels of energy production in their basal state, but they do not have a wide margin of reserve mitochondrial capacity to use in stress-inducing environments (22).

Physiological challenges such as changes in the surrounding environment or activation by agonists require metabolic responses in platelets. To remain viable, platelets must select from available fuels and use the appropriate metabolic pathways to generate the necessary energy. Platelets that have inadequate energy substrates or are unable to perform glycolysis, oxidative phosphorylation, or other energy metabolism pathways are unable respond to activating signals (2). Multiple energy transduction pathways provide the necessary ATP for functional platelets; platelet activation and subsequent granule secretion derive their energy from both glycolysis and oxidative phosphorylation (3, 9). Inhibition of mitochondrial respiration by pharmacological blockers of the electron transport chain complexes has been shown to significantly diminish granule secretion and platelet aggregation (9).

In addition to energy production, adequate mitochondrial function is also critical to reactive oxygen species (ROS) production and calcium homeostasis (9). Intracellular ROS production by mitochondria within activated platelets serves as a critical second messenger (9). The activity and ultimate fate of platelets is controlled in part by ROS formation and redox reactions mediated by functional mitochondria (10). Platelet-produced ROS is associated with the propagation of platelet activation by inactivating nitric oxide and releasing platelet agonists such as ADP (24). The reactive oxygen species generated by mitochondria as a result of oxidative phosphorylation must be neutralized to

prevent excess accumulation which can directly damage the cell. As mentioned previously, the hexose monophosphate shunt uses glucose to produce intermediates, specifically ribose-5-phosphate, necessary for the biosynthesis of nucleotides, nucleic acids, and coenzymes (6). In addition, the hexose monophosphate shunt also contributes to stabilizing the redox state of the cell by generating NADPH; NADPH protects the cell from oxidative damage by providing electrons to generate reduced glutathione for detoxification of hydrogen peroxide, a reactive oxygen intermediate formed by the partial reduction of oxygen (6). Hydrogen peroxide not neutralized by glutathione may react with iron in the blood and produce hydroxyl radical which is known to cause direct cellular injury (6). The generation of reactive oxygen species as a result of energy metabolism, without detoxification, will contribute to the progressive damage of platelet structure and function. Mitochondrial generated reactive oxygen species play a critical role in platelet activation, but if left unbalanced, can contribute to damage that reduces platelet functionality.

### **Platelet Storage**

*In vivo*, damaged platelets are continuously removed and replaced; the life span of a platelet in the cardiovascular system is approximately 9 days (25). However, platelets drawn from donor blood and stored in plasma or platelet additive solutions are not able to regenerate healthy populations, due to their lack of DNA. Storage of platelets has been found to cause partial activation and/or loss of metabolic function which contributes to a gradual loss in their ability to aggregate (25). The sum of all injurious changes leading to morphological and functional changes in platelets is collectively referred to as the Platelet Storage Lesion (PSL) (25). Although the exact mechanism of PSL has not been fully

elucidated, previous studies have reported changes in platelet morphology, activation, cell metabolism, and apoptosis during storage (2, 25). Changes in morphology include loss of discoid shape and abnormal forms (25). In an attempt to prevent the formation of the PSL by depressing metabolic activity, platelets were stored at cold temperatures (26). Electron microscopy revealed that platelets stored at cold temperatures experienced changes in morphology from a discoid to spherical shape with spiny projections on the surface; this altered morphology is similar to activated platelets. (25). Ultimately freezing reduced maximum storage time due to the formation of vWF receptor complex clusters that bind integrin receptors on liver macrophages resulting in rapid platelet clearance from circulation (25). In a study of platelet vitality at various storage temperatures, platelet half-life drops by more than 50% when stored at temperatures below 13°C; upon transfusion, the half-life of platelets in plasma drops from nine to less than five days when platelets are stored at temperatures below 13°C (25).

An alternative approach to avoid induction of the PSL is to store platelets in storage containers that allow for increased gas permeability to facilitate oxidative phosphorylation (25). Metabolic balance in stored platelets is due in part to the gas permeability of the container and the buffering capabilities of the media (2). Previous studies have found that platelets stored in gas impermeable bags had increased levels of anaerobic glycolysis leading to the accumulation of lactic acid and a subsequent drop in pH (25). Recent reports have documented changes in platelet morphology when the pH within the cell drops below 6.8 and a loss of viability is apparent when pH reaches 6.0 (25). A storage solution with adequate buffering capacity can help to minimize changes in pH as a result of metabolic imbalances due to decreased oxygen availability. To

facilitate equal oxygen diffusion and utilization within the storage container, platelets should be stored in gas permeable bags on a continuous agitator (25).

To extend the storage life of platelet concentrates beyond five days, platelets must be suspended in a solution that will provide the necessary fuels and buffering. Platelet additive solutions were first developed in the 1980s to support platelet metabolism during storage in reduced levels of plasma, 30-40% (1). Reducing the required plasma for platelet storage increases the amount of plasma available for fractionation and reduces the likelihood of transfusion-related complications (1). Platelet additive solutions currently used contain varying combinations and concentrations of citrate, phosphate, acetate, and glucose (1). Citrate is a weak calcium ion chelator and is used as an anticoagulant. Phosphate is added for its pH buffering capacity. Acetate and glucose are used as substrates for aerobic respiration (1). Metabolism of acetate and citrate through the TCA cycle generates carbon dioxide which is used as a source of bicarbonate ions for additional buffering capacity and pH stabilization (1).

Recent studies have demonstrated that the addition of glucose at concentrations ranging from 13.75 to 55 mM to platelet additive solutions significantly improves metabolism, survival and morphology of stored platelets (27). Supplementing platelet additive solutions with high glucose concentrations may exacerbate the accumulation of lactate production by glycolysis if the concentration of glucose overwhelms oxidative phosphorylation machinery (27). To avoid production of lactate, alternative fuels can be used instead of high glucose concentrations. Glutamine has been demonstrated as an effective substrate for mitochondrial respiration in platelets (28). Glutamine is metabolized by platelets into glutamate, aspartate, and CO<sub>2</sub> (28). Unlike glucose,

glutamine supplementation does not result in any lactate formation and although the potential energetic value of glutamine is less than that of glucose, Vasta *et al* demonstrated that glutamine is preferentially selected as a substrate for respiration over glucose (28). Hyperglycemia can also stimulate production of reactive oxygen species by platelet mitochondria, potentially negatively affecting platelet energy metabolism and its roles in hemostasis (29).

A potentially more efficient means for providing acetate as a substrate is through the addition of L-carnitine to storage solutions. Once transported into the cell, acetyl-carnitine directly reacts with CoA to produce acetylCoA and carnitine. Deyhim *et al* used L-carnitine in a series of pilot studies to explore the effects of different concentrations on platelet concentrate quality. They reported that 15mM of L-carnitine significantly decreased LDH activity, increased aggregation efficiency, and mitochondrial metabolic activity was better maintained compared to lower doses of L-carnitine (30).

Malfunctioning metabolic machinery that limits energy metabolism of glucose could be circumvented through the addition of exogenous sources of pyruvate and acetyl-CoA. While the inclusion of pyruvate as an alternative biofuel circumvents any malfunctioning glycolytic machinery, inclusion of pyruvate as an alternative biofuel could increase lactate formation by lactate dehydrogenase. Indeed, a study by Readnower *et al*, reported that pyruvate increased extracellular acidification rate (ECAR), primarily due to lactic acid production, following oligomycin and FCCP addition in adult mouse cardiomyocytes (31). Addition of fatty acids eliminates the need for glycolysis during energy metabolism and subsequently limits the production of lactate in stored platelet concentrates. Although many studies have reported the individual effects of alternative

biofuels, none have explored the potential synergistic effects of combining multiple substrates in a single platelet additive solution.

### **Apoptosis in Anucleate Platelets**

Stored platelets that lack substrates for energy metabolism or do not have proper gas exchange exhibit non-nuclear apoptosis; manifesting as a decrease in mean platelet volume (25). A number of studies have demonstrated induction of apoptosis in platelets exposed to very high shear stress, *in vivo* in circulation in canine and rodent models, and stored *in vitro* at 4, 22, and 37°C (32). Mitochondria-dependent apoptosis in platelets includes a number of classic parameters including membrane depolarization, formation of the mitochondrial permeability transition pore, expression, activation, and translocation of pro-apoptotic Bcl-2 proteins to the mitochondria, release of apoptogenic cytochrome c from the mitochondrial intermembrane space, and the activation of caspase-9 (32). Formation of the mitochondrial permeability transition pore allows cytochrome C to be released into the cytosol where it binds to apoptotic protease-activating factor 1 (Apaf-1), forming the apoptosome (32). The newly created apoptosome recruits and activates procaspase-9 which in turn initiates a caspase cascade involving caspases 3 and 7 (32). A recent study by Perrotta, *et al.* demonstrated that caspase-3 is cleaved during platelet activation and is responsible for platelet membrane blebbing and phosphatidylserine exposure (33). In addition to caspase-3, cleaved caspase-7 also contributes to the morphologic and biochemical changes associated with apoptosis in platelets, including cell shrinkage and membrane microvesiculation (33).

Upon activation, intentionally by the coagulation cascade or unintentionally as a consequence of storage, phosphatidylserine will be exposed on the outer membrane;

phosphatidylserine exposure signals the cell to initiate apoptosis (33). Annexin V staining can be used to identify apoptotic cells. Annexins are a family of calcium-dependent phospholipid-binding proteins that bind to phosphatidylserine. In healthy cells, phosphatidylserine is predominantly located on the cytosolic side of the plasma membrane. Translocation of phosphatidylserine to the extracellular membrane is detectable using fluorescently labeled Annexin V.

Preventing premature apoptosis in stored platelets could potentially elongate the storage life of platelet concentrates. Previous studies using various cell types have demonstrated that antioxidants can reduce apoptosis (34, 35). The addition of antioxidants such as glutathione, reduced coenzyme Q, and epigallocatechin-3-gallate to storage mediums may prolong platelet storage time by minimizing apoptosis (34, 36).

Surprisingly, platelets that are not fully functional *in vitro* are able to recover their function *in vivo* (2, 25). Fuels that are depleted in a closed storage system are replenished when the platelets return to an *in vivo* environment and toxic metabolites that had accumulated in the storage container are diluted in the circulatory system (25). Rinder, *et al.* investigated the potential reversibility of metabolic stress in stored platelets. They found that metabolic and pH abnormalities observed in stored platelets can be reversed by restoring optimal pH and fuel levels to the storage media (37). To assure that any loss in platelet function *in vitro* due to storage conditions is reversible upon transfusion, we will attempt to maintain at least 80% of the initial platelet count and responsiveness to activation beyond five days of storage.

## **Extracellular Flux Analysis of Cellular Bioenergetics**

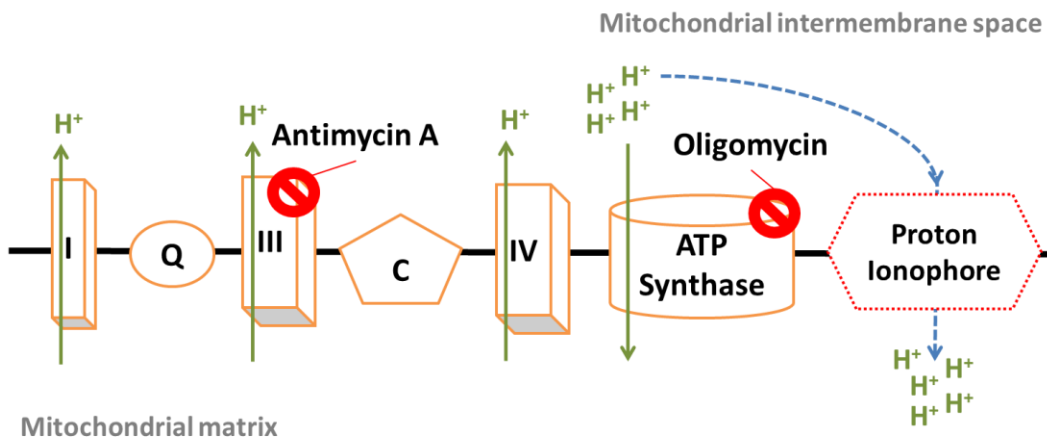
Cellular bioenergetics can be assessed using numerous methods including blood gas analyzers, Clark-type oxygen electrodes, high-resolution respirometry, and extracellular flux analyzers. Blood gas analysis can be used to measure pH,  $pO_2$ ,  $pCO_2$ ,  $HCO_3^-$ , and glucose content. Oxygen tension is used to calculate the oxygen consumption rate and evaluate oxidative phosphorylation activity. Changes in pH provide an indication of lactate production by glycolysis. Alternatively, Clark-type electrodes can be used to measure oxygen consumption, but the continuous consumption of oxygen during the procedure decreases oxygen pressure in the cells. In addition, Clark-type electrodes are unable to measure glycolysis. Both high-resolution respirometry and extracellular flux analyzers allow real-time analysis of mitochondrial function in live cells.

The XF24 Extracellular Flux Analyzer by Seahorse Bioscience was selected over the Oroboros O2k high resolution respirometer for its fully automated system, high-throughput capabilities, and minimal time per sample assay. The XF24 Extracellular Flux Analyzer can be used to analyze mitochondrial respiration and glycolytic acidification in live intact cells. Real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are made by isolating a small volume, 7  $\mu$ L, above a single layer of cells within a microplate. Solid state sensor probes residing above the cell layer are used to measure cellular oxygen consumption and lactic acid production within the transient microchamber. The fluorescent probes are able to accurately measure changes in concentrations of dissolved oxygen and free protons due to the high cell to volume ratio created by the transient microchamber. The changes in dissolved oxygen and free protons are used to determine the determine OCR (pMoles/min) and ECAR

(mpH/min) during a specified period of time. Once the measurement is complete, the probes raise to allow the larger medium to mix with the medium in the transient microchamber and cell values are restored to baseline. After mixing, measurements are recorded several times.

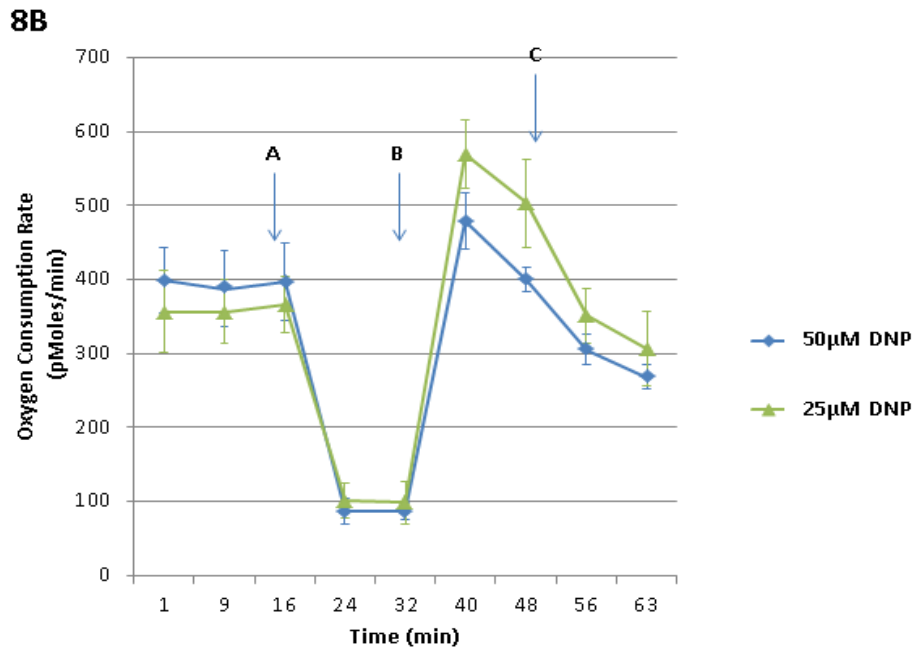
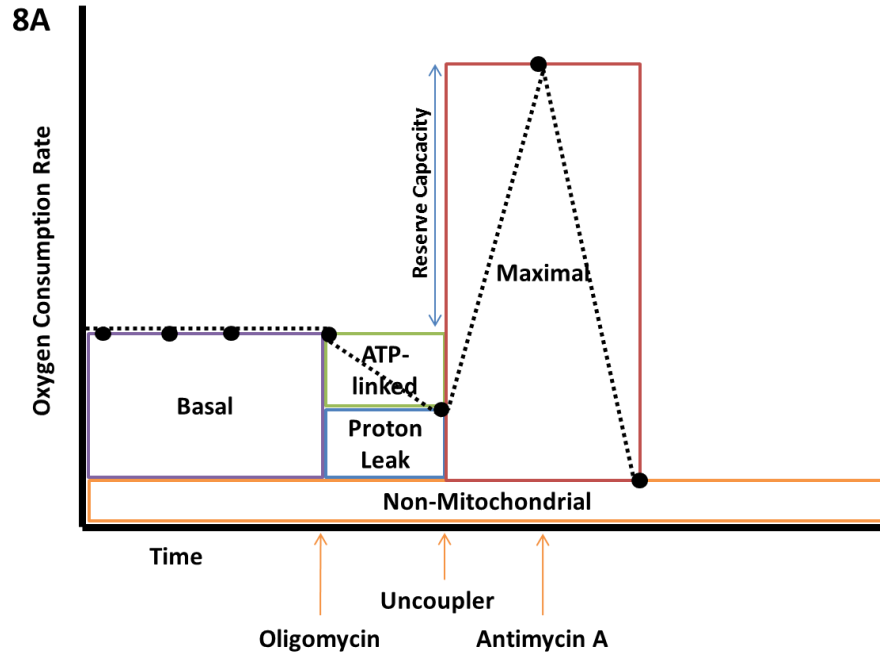
Mitochondrial bioenergetic function is analyzed by sequentially adding pharmacological modulators of oxidative phosphorylation (See Figure 7, 8) (15). The mean basal respiration is determined by taking multiple sequential measurements prior to the injection of any mitochondrially targeted drugs. Basal respiration requires the proton motive force to generate ATP or move ions across the inner membrane; pharmacological inhibition of energy-requiring processes results in a decrease in basal respiration (15). Under this assumption, oligomycin, an ATP synthase inhibitor, can be used to determine the proportion of ATP-linked OCR and proton leak. Inhibition of ATP synthase by oligomycin results in a decrease in OCR representing the rate of oxygen consumption corresponding to ATP synthesis (22). The oligomycin-insensitive rate is caused by protons leaking across the inner mitochondrial membrane during oxidative phosphorylation (22). To estimate the maximal respiration attainable, a proton ionophore is injected. Exposure to a proton ionophore (uncoupler) dramatically increases permeability of the mitochondrial inner membrane to protons. The uncoupler dissolves in the phospholipid bilayer and increases the permeability of the membrane to protons. Within the membrane, uncouplers shield the electric charge as protons pass through the membrane and effectively disconnect the electron transport chain from the formation of ATP. Consequently, electron transfer is no longer regulated by the proton gradient and oxygen consumption increases. The resulting oxygen consumption rate is the maximum

achieved at Cytochrome C oxidase regardless of the availability of substrates or electron transport chain activities. The addition of an uncoupler also allows one to determine the reserve capacity. The reserve capacity is a measure of spare respiratory capacity, most commonly used in stress-inducing environments. It is calculated as the difference between the basal rate and the uncoupler-stimulated maximum respiration (22). Finally, pharmacological inhibition of a component of the electron transport chain allows for determination of non-mitochondrial oxygen consuming processes (15). Antimycin A, a complex III inhibitor, obstructs the majority of oxygen consumption in the mitochondria and any remaining oxygen consumption is attributed to non-mitochondrial oxidases (15, 22).



**Figure 7: Inhibitors of the Electron Transport System**

Proton Ionophores dramatically increase the permeability of the mitochondrial inner membrane to protons. Antimycin A obstructs the majority of oxygen consumption in the mitochondria by directly inhibiting Complex III. Oligomycin directly inhibits ATP Synthase, allowing for the measurement of oxygen consumption rate dependent on ATP generation.



**Figure 8: Bioenergetic Profile Generated Using Inhibitors of the Electron Transport Chain**

(A) A theoretical bioenergetic profile: after establishing the basal oxygen consumption rate, Oligomycin is injected. The decrease in oxygen consumption rate indicates the proportion of oxygen consumption dependent on ATP generation. Addition of an uncoupler, or proton ionophore, stimulates maximum respiration by removing the rate limiting capacity of ATP Synthase. Antimycin A directly inhibits Complex III allowing for measurement of respiration not linked to mitochondrial activities. (B) Platelets isolated from a healthy adult donor were plated at  $6 \times 10^7$  platelets per well. Each data point represents the average of three independent wells. Oxygen consumption rate (OCR) is plotted against time (minutes). Injection site A represents the addition of 2.5  $\mu$ M oligomycin. Injection sites B and C represent the addition of the designated concentration of DNP.

One of the best measurements of healthy mitochondrial activity is the Respiratory Control Ratio (RCR), which is defined as the respiration in state 3 divided by that in state 4. Healthy mitochondria have a high respiratory control ratio, with a large increase in respiration rate upon addition of ADP (State 3) followed by a gradual return to resting (State 4) respiration (38). The RCR is an indication of the mitochondria's ability to idle at a low rate but also respond quickly to ADP; a high RCR indicates that the mitochondria have a high capacity for substrate oxidation and ATP turnover, and low proton leak (38).

### **Optimization of Extracellular Flux Analysis of Human Platelets**

Several studies have published methods for isolating and defining distinct bioenergetic profiles in blood cells in hope of using blood cells as a diagnostic tool to assess the role of mitochondrial dysfunction in several pathologies (15, 16, 22, 39). To obtain the unique bioenergetic profile of platelets, whole blood must be separated into its components. If extracellular flux analysis was performed on a sample of whole blood, the results would be an average of the different bioenergetic profiles of monocytes, lymphocytes, neutrophils, and platelets (16). Establishing a reliable and reproducible basal bioenergetic profile for platelets is a critical component of achieving our principal objective. It will allow us to compare different storage mediums and to assess mitochondrial dysfunction in different patient populations. After establishing a reliable and reproducible method to assess bioenergetic function in intact platelets, we will evaluate the effectiveness of different alternative biofuels, antioxidants, and other mitochondrial drugs in platelet storage solutions to extend the lifespan of stored platelet concentrates. We will also work to elucidate the relationship between platelet oxidative

phosphorylation, anaerobic glycolysis, ATP levels, and oxidative stress during extended storage times.

## **Research Approach & Methodology**

The studies described herein were approved by the University of Maryland, Baltimore Institutional Review Board and all participants provided written informed consent.

### **Platelet Isolation from Whole Blood**

To determine the bioenergetic activity of platelets, platelets must be separated from other whole blood components. Using a crude preparation of whole blood would result in a weighted average of the bioenergetic activity of multiple cell populations found within the blood (16). Platelets were isolated from whole blood by differential centrifugation. We achieved an average isolation yield above 70% using an initial centrifugation speed of 200g for 10 minutes to separate the red blood cells from the plasma rich plasma, followed by a 1600g spin for 10 minutes to pellet the platelets. Platelets were then resuspended in an equal volume of the running buffer to the plasma volume.

### **Determination of Platelet Count**

To obtain the platelet count from a sample of whole blood, a 1:25 dilution of whole blood to Isoton II Diluent was prepared and centrifuged for 90 seconds at 600g. 500  $\mu$ L of the platelet-rich supernatant was removed and added to 10 mL of Isoton II Diluent to achieve a final dilution of 1:500. To determine the platelet concentration in a sample of isolated platelets suspended in media, a 1:1000 dilution of platelet concentrate to Isoton II Diluent was used. The concentration of platelets per  $\mu$ L was determined by dividing the reported count by the metered volume and then multiplying by the dilution factor.

### **Preparation of Seahorse Running Buffer**

Seahorse Bioscience recommends the use of the XF Base Medium for all extracellular flux assays; the base medium is formulated without buffering agents, glucose, glutamine, or sodium pyruvate to allow the user to customize the assay medium. XF assays require a lightly buffered medium to sensitively measure the extracellular acidification rate. The base running buffer used in all experiments, unless noted otherwise, was prepared by dissolving 8.3 g of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma D5030) into 990 mL of sterile water, pre-warmed to 37°C. 1.85 g of NaCl (Sigma 71376) was added to achieve a final concentration of 143 mM. The base medium was sterile filtered and then divided into smaller aliquots and frozen until needed. Prior to each experiment, glucose, glutamine, and pyruvate are added to the media as substrates for energy metabolism. In addition, an anticoagulant is added to prevent coagulation of platelets during processing. Finally, the solution was adjusted to pH 7.4 in accordance with Seahorse Bioscience recommendations. A neutral pH environment is essential for accurate measurements of oxygen consumption and extracellular acidification rate; pH changes in the media during the assay can affect mitochondrial bioenergetics.

### **Preparation and Calibration of Seahorse Sensor Cartridge**

Before each experiment, 1 mL of XF Calibrant Solution (Seahorse Bioscience) was added to each well of the 24 well dual-analyzer sensor cartridge (Seahorse Bioscience). The sensor cartridge and calibration plate were incubated at 37°C without CO<sub>2</sub> for a minimum of 4 hours, usually overnight. The day of the experiment, the injection ports on the sensor cartridge are loaded with the experimental reagents and placed into the Seahorse XF24 Flux Analyzer for automated calibration. The calibration

procedure performed at the beginning of each assay is necessary to establish the fluorescence values for each sensor that correspond to known concentration of O<sub>2</sub> and H<sup>+</sup> in the calibration solution.

### **Platelet Seeding**

Prior to the XF assay, platelets must be immobilized onto the XF cell culture microplate. Corning<sup>®</sup> Cell-Tak<sup>™</sup> is used as an adhesive for cells in suspension that do not naturally settle on the bottom of a microplate well. Each well was coated with 3.5 µg/cm<sup>2</sup> of Cell-Tak. After the Cell-Tak absorbs into the microplate and the plate is washed once with ultrapure water to remove excess Cell-tak, platelets are added to each well at the desired concentration in 200 µL of running buffer. The microplate was then placed in a 37°C incubator with no CO<sub>2</sub> for 30 minutes. After the incubation period, the microplate was centrifuged to encourage the cells to strongly adhere to the bottom of the microplate. The optimal centrifugation speed was determined to be 1600g for 5 minutes with no brake. The cell plate was then rotated 180° before spinning at 1600g for an additional 5 minutes with no brake. After centrifugation, additional running buffer was added to achieve a final volume of 675 µL per well. Plates were incubated for an additional 45-60 minutes at 37°C and then placed into the calibrated Seahorse XF24 flux analyzer for mitochondrial bioenergetic analysis.

### **Preparation of Mitochondrial Substrates and Inhibitors**

Stocks of mitochondrial substrates were prepared as follows and stored at -20°C until use. 24 mM Oligomycin stock was prepared by dissolving 10 mg Oligomycin (Calbiochem 495455) in 526 µL 95% ethanol. 24 mM Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) stock was prepared by dissolving 10mg of FCCP (Sigma C2920) in 1.6 mL 95% ethanol. A 13mM Antimycin A stock was prepared

by dissolving 25mg of Antimycin A from Streptomyces (Sigma A8674) in 3.6 mL 95% ethanol. 50mM 2,4-Dinitrophenol (DNP) was prepared by dissolving 5g of DNP (Sigma D198501) in 543 mL Dimethyl sulfoxide (Sigma 472301). The 50 mM DNP solution was then diluted with ultrapure water to achieve a stock of 3 mM with pH of 7.7.

### **Establishing a Baseline Bioenergetic Profile for Platelets**

As discussed previously, real-time measurements of the mitochondrial bioenergetics of isolated platelets were determined using an extracellular flux analyzer. Four injection ports are available in each well for the sequential addition of inhibitors of mitochondria respiratory chain or activators of the oxidative burst to determine alterations in cellular respiration pathways. Pilot experiments were performed to determine the optimal platelet concentration for accurate measurements. The optimum concentration of mitochondrial drugs was determined by titration of each individual compound.

### **Analysis of Data Generated by Seahorse Bioscience XF24**

As described previously by Sauerbeck *et al.*, for optimal analysis of mitochondrial bioenergetic function, data generated by the XF analyzer should be analyzed beyond the average oxygen consumption ratio generated for a given measure. During any given measurement, the rate of oxygen consumption will vary with time as substrates are consumed by the mitochondria. Using the Microsoft Excel software package plug-in offered by Seahorse Bioscience, point-by-point rates of oxygen tension and pH can be collected and analyzed.

### **Determination of Protein Content**

Seahorse data collected was normalized by protein content to correct for fluctuations in plating efficiency. Protein content per well was determined using a Micro

BCA Protein Assay kit (Thermo Scientific 23235). After the XF assay was complete, the supernatant was removed from each well. A 10% RIPA Buffer (Life Technologies 89900) containing 1% of protease inhibitor cocktail (EMD Millipore 535140) was added to each well. The RIPA buffer used to lyse cells contains buffering agents, chelators, and detergents. The protease inhibitor cocktail is used to protect the integrity of proteins during protein extraction and purification. A micropipette was used to manually detach platelets attached to the bottom of the well. Samples from each well were collected and stored until the Micro BCA Assay was performed.

### **Analysis of Platelet Bioenergetics in Cardiopulmonary Bypass Patients**

Whole blood samples were collected from patients immediately before and following cardiopulmonary bypass. Platelets were isolated from whole blood samples using the techniques previously described. Platelets were plated at  $3 \times 10^7$  or  $6 \times 10^7$  depending on the patient's total platelet count. Platelet seeding was accomplished using two different techniques. The first four patients' platelets were seeded using a 140g centrifugation speed for 10 minutes. All subsequent patients' platelets were seeded using the newly optimized centrifugation technique featuring a 1600g centrifugation for 5 minutes with no brake. The plate was then rotated 180° and centrifuged for an additional 5 minutes at the same speed. Maximum oxygen consumption rate was calculated for each patient before and after surgery normalized to  $\mu\text{g}$  of protein. Respiratory control ratio was calculated using the normalized oxygen consumption rates.

### **Analysis of Platelet Bioenergetics in Stroke Patients Suffering from Depression**

Whole blood samples were collected from patients. Platelets were isolated from whole blood samples using the techniques previously described. Platelets were plated at 6

$\times 10^7$  per well. Platelet seeding was accomplished using a 1600g centrifugation for 5 minutes with no brake. The plate was then rotated 180° and centrifuged for an additional 5 minutes at the same speed. The bioenergetic profile for each patient was normalized to  $\mu\text{g}$  of protein.

## **Outcomes of Research**

### **Optimization of Isolation of Platelets from Whole Blood**

As stated previously, isolation of platelets from whole blood is necessary to determine the bioenergetic profile of platelets. Platelet rich plasma is prepared by differential centrifugation; the acceleration force is adjusted to sediment certain cellular components based on their different specific gravities (40). Previously published protocols have been optimized with respect to volume of initial whole blood sample, time period of centrifugation, and range of centrifugal acceleration (40). Current literature recommendations for separating red blood cells from the platelet rich plasma range from 150-500g for 10-20 minutes (16, 22, 41, 42). A second centrifugation step is necessary to pellet the platelets and remove any interfering substances suspended within the plasma; the current literature suggests 1500g for approximately 10 minutes (16, 22, 41, 42). Initially we used a 500g spin for 12 minutes to separate the red blood cells from platelet rich plasma, followed by a 1600g spin for 10 minutes to pellet the platelets; resulting in a 30% yield. The initial 500g spin may have activated the platelets, causing them to aggregate. Aggregated platelets are more dense than their inactive counterparts and will be pulled into the red blood cell layer during differential centrifugation. We hypothesized that a softer initial spin was necessary to prevent platelet activation and improve total yield. After testing numerous speeds and times based on literature recommendations, we found that we could achieve an average isolation yield above 75% using an initial centrifugation speed of 200g for 10 minutes to separate the red blood cells from the plasma rich plasma, followed by a 1600g spin for 10 minutes to pellet the platelets. Platelets were then resuspended in an equal volume of the running buffer to the plasma volume.

## **Assessing the reliability of the Z1 Coulter Counter for analyzing platelet concentrations**

Platelet count in whole blood and isolated samples was determined using a Beckman Coulter Z1 Coulter Counter Single Threshold Particle Counter. The Z1 Coulter Counter uses impedance technology, the Coulter Principle, to count particles in the 1-120  $\mu\text{M}$  range. A small, 50 $\mu\text{m}$  aperture is immersed into a beaker containing the sample diluted in a low concentration electrolyte solution. An electrical current is applied between two electrodes, one inside the aperture tube and another inside the sample beaker. The particles suspended in the electrolyte solution are counted as they pass through the aperture and an equal volume of electrolyte solution is displaced from the sensing zone causing a short-term change in the impedance across the aperture. To determine the concentration of platelets in a given sample of whole blood or isolated platelets, size restrictions recommended by Beckman Coulter were placed upon the measurements; only those particles between 3-30 fL were measured. To obtain the platelet count from a sample of whole blood, a 1:25 dilution of whole blood to Isoton II Diluent was prepared and centrifuged for 90 seconds at 600g. 500  $\mu\text{L}$  of the platelet-rich supernatant was removed and added to 10 mL of Isoton II Diluent to achieve a final dilution of 1:500. To determine the platelet concentration in a sample of isolated platelets suspended in media, a 1:1000 dilution of platelet concentrate to Isoton II Diluent was used. The concentration of platelets per  $\mu\text{L}$  was determined by dividing the reported count by the metered volume and then multiplying by the dilution factor.

To ensure that the Coulter Counter produces precise measurements of platelets in both whole blood and isolated platelets, three measurements are recorded for each sample and then the average is used for subsequent calculations. Whole blood samples were

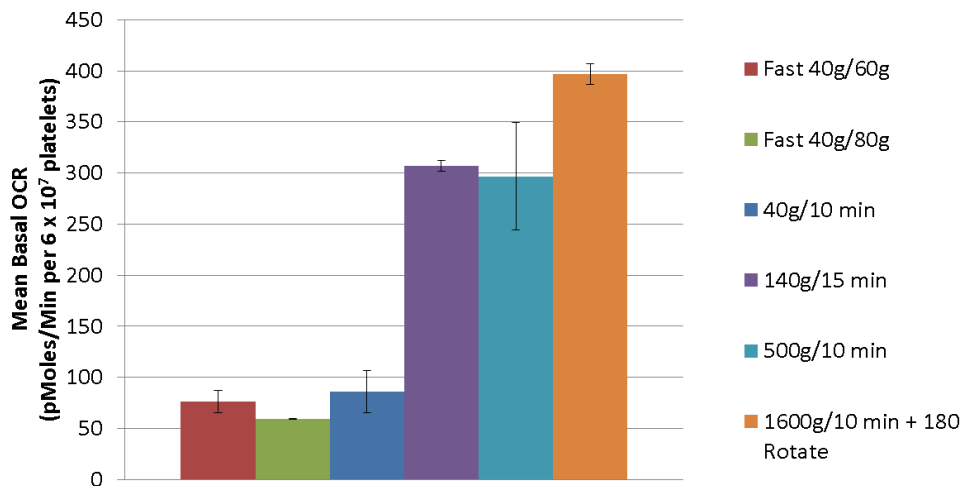
collected from the same volunteer during a five month period. Assuming that the patient's platelet count did not vary dramatically between each donation, the standard error among triplicate measurements on a given day can be calculated and used as an indication of the Coulter Counter's reliability. During a five month period, the patient's platelet count in a sample of whole blood, measured by the Coulter Counter, ranged from approximately 179,000 and 260,000 platelets per microliter, with an average of 214,780 platelets per microliter. Measurements were recorded in triplicates for each whole blood sample on any given donation date. The average standard error was 942.7 per microliter. The average standard error is less than 1% of the average platelet count on a given date, verifying that the Coulter Counter provides precise measurements of platelets in whole blood samples. The same procedure was used to assess the Coulter Counter's ability to produce precise measurements of isolated platelets. The average isolated platelet count ranged from approximately 450,000 – 765,000 platelets per microliter with a standard error of 2,779.1; the average standard error is less than 1% of the average platelet count on a given date, verifying that the Coulter Counter provides precise measurements of platelets in isolated platelet samples.

### **Platelet Seeding Optimization**

As discussed previously, immobilization of the platelets on the XF cell culture microplate is critical for success of the XF assay. After coating the microplate with Corning® Cell-Tak™, the microplate is centrifuged to encourage the cells to strongly adhere to the bottom of the microplate. After centrifugation, the final well volume is adjusted to 675  $\mu$ L. Current literature recommendations for centrifugation speeds for platelet seeding range from 40g to 1000g (16, 22). To avoid incidental activation during

centrifugation, we attempted to attach the platelets to the bottom of the plate by centrifuging at the lowest recommended speed, 40g (22). Three different durations were used at 40g. In our initial experiment, labeled Fast 40g/60g, once the centrifuge achieved 40g it was stopped without brake application. The centrifuge was then set to 60g and the process was repeated. The second experiment, labeled as Fast 40g/80g, used the same procedure but the second centrifugation speed used was 80g instead of 60g. In a third experiment, the centrifugation speed was maintained at 40g for 10 minutes. These attempts using low centrifugation speeds yielded low mean basal OCR which prompted us to increase the centrifugation speed to encourage stronger seeding. Subsequent experiments increased the speed and duration of centrifugation to 140g for 15 minutes, 500g for 10 minutes, and 1600g for 10 minutes. After centrifugation, the final volume of each well is increased to 675  $\mu$ L.

Platelet seeding efficiency was measured as the mean basal OCR (pMoles/min per  $6.0 \times 10^7$  platelets), where the mean basal OCR is the average of at least three independent wells. Centrifugation at 1600g for 10 minutes with a 180° rotation midway produced the highest average basal OCR with the narrowest spread of values, 396.9 ( $\pm$  10.2) pMoles/min per  $6.0 \times 10^7$  platelets (Refer to Figure 9).



**Figure 9: Seeding Centrifugation Optimization**

Platelet seeding efficiency was measured as the mean basal oxygen consumption rate  $\pm$  standard error (pMoles/min) per  $6.0 \times 10^7$  platelets. For all conditions,  $n = 3-9$  independent wells. Centrifugation techniques are described as follows: Centrifuge the plate at 40g for 1 second with no brake, rotate the plate  $180^\circ$  and centrifuge again with no brake at 60g for 1 second (labeled Fast 40g/60g). Centrifuge the plate at 40g for 1 second with no brake, rotate the plate  $180^\circ$  and centrifuge again with no brake at 80g for 1 second (labeled Fast 40g/80g). Centrifuge the plate at 40g for 10 minutes (labeled 40g/10 min). Centrifuge the plate at 140g for 15 minutes (labeled 140g/15 min). Centrifuge the plate at 500g for 10 minutes (labeled 500g/10 min). Centrifuge the plate at 1600g for 5 minutes with no brake, rotate the plate  $180^\circ$  and centrifuge again with no brake at 1600g for 5 minutes (labeled 1600g/10 min + 180 rotate).

Platelets that are not firmly attached to the bottom of the plate may be dislodged when the well volume is adjusted to  $675\mu\text{L}$ ; lack of immobilization to the bottom of the well may allow platelets suspended in media to be displaced outside of the microenvironment created by the lowered probe and therefore out of the measurable range. Low basal OCR may be representative of a reduced population of platelets in the microenvironment created by the lowered probe during measurement. During the mixing phase of the protocol, the probe is raised to allow the well to re-equilibrate. Once the probe is lowered again to measure oxygen consumption and lactate production, a new microenvironment is created with a potentially different platelet population. This creates

variability among the data collected from each well. As expected, platelet seeding techniques using slow centrifugation speeds resulted in increased variability among measurements as the microenvironment created by the probes varied throughout the assay. Using a fast 1600g spin with a 180° rotation allowed for a monolayer of platelets to firmly adhere to the microplate.

### **Titration of Mitochondrial Drugs**

In addition to provoking inconsistencies in regards to the oxygen consumption rate, fluctuating platelet populations within the transient microchamber may not respond appropriately to previously titrated mitochondrial drugs. Once the optimal platelet seeding technique was selected, pilot experiments were performed to determine the optimum cell number required for accurate measurements; the optimal platelet seeding density was determined to be  $6 \times 10^7$  cells per well. The average maximum oxygen consumption rate for  $6 \times 10^7$  platelets per well was 396.9 ( $\pm 10.2$ ) pMoles/min with an average 81.2 ( $\pm 2.1$ )  $\mu\text{g}$  protein/well. Other groups have recommended lower optimal seeding densities for platelets, but we found that a higher seeding density allowed for a greater respiratory control ratio. A high respiratory control ratio will allow for more sensitive measurements of fluctuations in different storage media or patient populations.

The optimum concentration of mitochondrial inhibitors was determined by titrating individual compounds in separate experiments against the cell number determined in the first set of experiments. Data is not shown for all pilot experiments; Table 1 below is provided as an example. The average maximum oxygen consumption rate and respiratory control rate using various concentrations of DNP in the presence of 2.5 $\mu\text{M}$  of Oligomycin for  $6 \times 10^7$  platelets per well are reported. The mean maximum

oxygen consumption rate is representative of the average of triplicate wells in duplicate assays. Two sequential additions of 15  $\mu\text{M}$  2,4 DNP yielded a mean maximum respiration of 7.33 ( $\pm$  0.88) pMoles/min/ $\mu\text{g}$  protein. Based on these results, future experiments will utilize two sequential additions of 15  $\mu\text{M}$  2,4 DNP to stimulate maximum oxygen consumption. Two sequential additions of 2,4 DNP are necessary to ensure that proton flow stimulated by the uncoupler exceeds the rate at which ATP synthase utilizes protons to produce ATP.

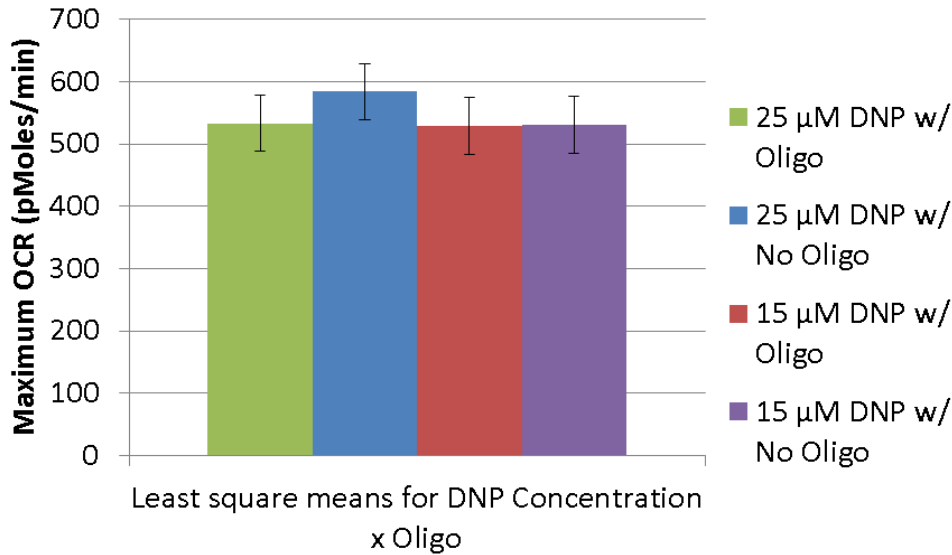
<b>2,4 DNP Concentration</b>	<b>Mean Maximum Respiration (pMoles/min/<math>\mu\text{g}</math> protein)</b>	<b>Std Error</b>
15 $\mu\text{M}$	7.33	0.88
25 $\mu\text{M}$	7.44	1.08
50 $\mu\text{M}$	4.93	0.75

**Table 1: Mean maximum oxygen consumption rate (pMoles/min/ $\mu\text{g}$  protein) for 15-200 $\mu\text{M}$  of 2,4 DNP**  
Mean maximum oxygen consumption rate (pMoles/min/ $\mu\text{g}$  protein)  $\pm$  standard error is reported from varying concentrations of 2,4 DNP (n=3-4). The mean maximum oxygen consumption rate is representative of the average of triplicate wells in duplicate assays. Two sequential additions of 15  $\mu\text{M}$  2,4 DNP yielded a mean maximum respiration of 7.33 pMoles/min/ $\mu\text{g}$  protein  $\pm$  0.88.

### **Effect of Oligomycin on DNP-stimulated Maximum Respiration**

Protonophores such as FCCP and 2,4 DNP dissipate the hydrogen ion gradient across the mitochondrial inner membrane, uncoupling electron transport from oxidative phosphorylation (43). Once uncoupled, ATP production is no longer linked to ATP synthase activity. Therefore, one would expect that the presence of an ATP Synthase Inhibitor, such as oligomycin, should have no effect on the maximum respiration stimulated by an uncoupler. However, Chappell, *et al.* published a study in 1961 on the effects of oligomycin on respiration and swelling in isolated liver mitochondria; they

reported that oligomycin inhibited mitochondrial DNP-stimulated adenosine triphosphate production (44). As a precaution, preliminary studies of the bioenergetic profile of healthy platelets required all treatment groups to be evaluated with and without the presence of oligomycin to obtain the maximum respiration unhampered by the presence of an ATP synthase inhibitor. Using a two-way ANOVA, the maximum oxygen consumption rate (pMoles/min per  $6.0 \times 10^7$  platelets) stimulated by 15  $\mu\text{M}$  or 25  $\mu\text{M}$  concentrations of 2,4 DNP was compared in the presence and absence of 2.5  $\mu\text{M}$  oligomycin ( $n = 4$ ) (Refer to Figure 10). The difference in the mean values among the different levels of oligomycin, 0 or 2.5  $\mu\text{M}$ , is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DNP Concentration; there is not a statistically significant difference ( $P = 0.567$ ). We also found that the effect of different levels of oligomycin does not depend on what concentration of DNP is present; there is not a statistically significant interaction between DNP Concentration and oligomycin. ( $P = 0.596$ ). As a result of these findings, future studies using 2,4 DNP as the protonophore for mitochondrial studies of platelets using a microplate based system does not necessitate each treatment group to be tested in the presence and absence of oligomycin.



**Figure 10: Maximum oxygen consumption rate (pMoles/min) per  $6 \times 10^7$  platelets for 15-25 μM 2,4 DNP with and without 2.5 μM Oligomycin**

Using a two-way ANOVA, the maximum oxygen consumption rate  $\pm$  standard error (pMoles/min) per  $6.0 \times 10^7$  platelets stimulated by 15 μM or 25 μM concentrations of 2,4 DNP was compared in the presence and absence of 2.5 μM oligomycin ( $n = 4$ ). The difference in the mean values among the different levels of oligomycin, 0 or 2.5 μM, is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DNP Concentration; there is not a statistically significant difference ( $P = 0.567$ ). We also found that the effect of different levels of oligomycin does not depend on what concentration of DNP is present; there is not a statistically significant interaction between DNP Concentration and oligomycin. ( $P = 0.596$ ).

### **Effect of Oligomycin and 2,4 DNP on Extracellular Acidification Rate**

In addition to measurements of oxygen consumption rate, the extracellular flux analyzer is also capable of measuring the extracellular acidification rate (mpH/min). The extracellular acidification rate is an indicator of protons released by glycolytic flux. As glycolysis increases, the rate of extracellular acidification is expected to increase. One would expect glycolytic flux to increase as cells attempt to recover mitochondrial ATP lost due to inhibition of oxidative phosphorylation. Measurements of ECAR provide an

indication of the contribution of glycolysis to the cellular bioenergetics of platelets. To investigate the contribution of glycolysis in platelet metabolism, inhibitors of oxidative phosphorylation are added.

Prior to the addition of any inhibitors of oxidative phosphorylation, the basal ECAR rate for platelets is established. In the presence of 5 mM Glucose, 4 mM Glutamine, and 1 mM Pyruvate, the basal ECAR rate is  $0.559 \pm 0.074$  mpH/min/ $\mu$ g protein. The addition of an ATP synthase inhibitor increases glycolytic flux to  $1.056 \pm 0.134$  mpH/min/ $\mu$ g protein. Increasing concentrations of 2,4 DNP, 15  $\mu$ M and 25  $\mu$ M, further stimulate proton production to  $1.858 \pm 0.212$  and  $2.095 \pm 0.247$  mpH/min/ $\mu$ g protein, respectively. As expected, increasing concentrations of an uncoupler increases glycolytic flux in platelets. These results confirm that inhibition of oxidative phosphorylation using ATP synthase inhibitor and proton ionophores shifts the metabolic paradigm in platelets to favor glycolysis as a mechanism for energy production.

### **Media Optimization**

To obtain the mitochondrial bioenergetic profile using an extracellular flux analyzer, isolated platelets are suspended in non-buffered media. The XF assay buffer recommended by Seahorse Bioscience and used by Chacko, *et al.* contains DMEM supplemented with 5.5 mM Glucose, 4 mM L-glutamine, and 1 mM Pyruvate (22). XF assays require a non-buffered medium to sensitively measure extracellular acidification rate (ECAR) of cells. Therefore, platelets suspended in plasma or storage solutions must be pelleted and resuspended in the XF assay buffer for analysis.

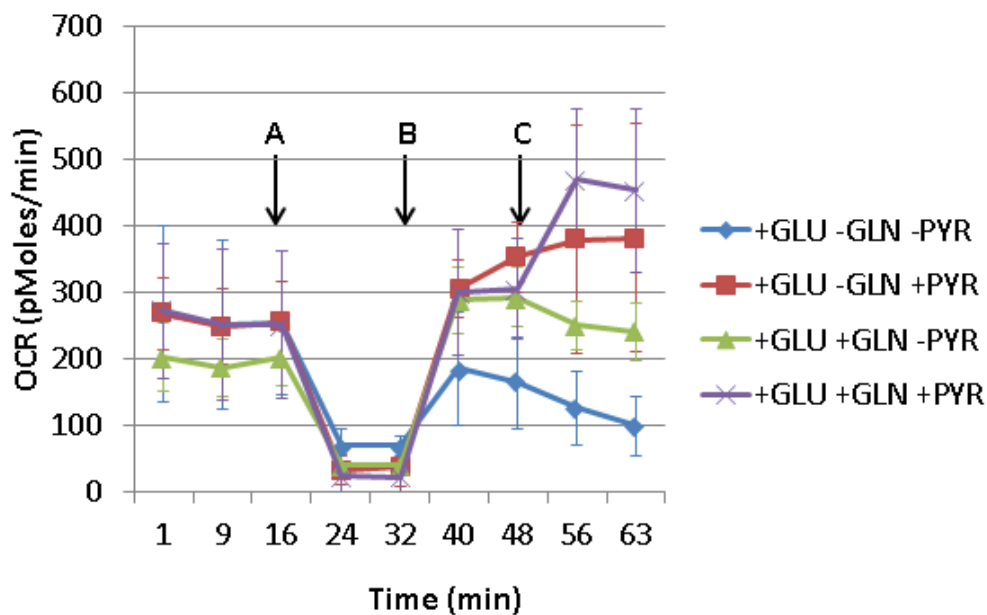
To prevent aggregation, an anticoagulant must be added to the media. Previous studies have used PGI<sub>2</sub>, heparin, citrate, and acid citrate dextrose as anticoagulants (16,

22, 39). We selected EDTA as an anticoagulant to maintain consistency throughout collection and processing. To confirm that EDTA was an appropriate anticoagulant for our study and would not interfere with measurements using the XF Flux Analyzer, we collected blood from a healthy donor into two separate containers, EDTA-coated vacutainers and citrate vacutainers. Blood from both collection containers was kept separate during the platelet isolation protocol. Platelets derived from blood collected in the EDTA-coated vacutainers was pelleted and resuspended in DMEM supplemented with 5.5 mM Glucose, 4 mM L-glutamine, 1 mM Pyruvate, with or without 6.2 mM EDTA. Platelets derived from blood collected in the citrate vacutainers was pelleted and resuspended in a citrate solution used frequently by the Red Cross as a storage solution; the storage media contains 15.5 mM Citric Acid, 89.4 mM Sodium Citrate, 16 mM Sodium Phosphate, and 257.5 mM Dextrose. Platelets resuspended in the supplemented DMEM lacking EDTA quickly activated and we were unable to perform the assay, demonstrating the necessity of an anticoagulant during measurements. Platelets exposed to EDTA as an anticoagulant, compared to citrate, had higher basal OCR and a higher RCR, 355 (pMoles/min per  $6 \times 10^7$  platelets) and 4.5, respectively. Values reported for each condition are the average of three independent wells. Platelets collected in citrate vacutainers had a lower basal OCR, 259 (pMoles/min per  $6 \times 10^7$  platelets) and a lower RCR, 2.6. Due to variations in substrate quantities between the supplemented DMEM and Red Cross citrate storage solution, it is unclear if the lower basal respiration and respiratory control ratio are due to the presence of citrate as an anticoagulant or hyperglycemic conditions. The high concentration of glucose in the citrate storage solution, 257 mM glucose, could potentially overwhelm the platelet's capacity to utilize it

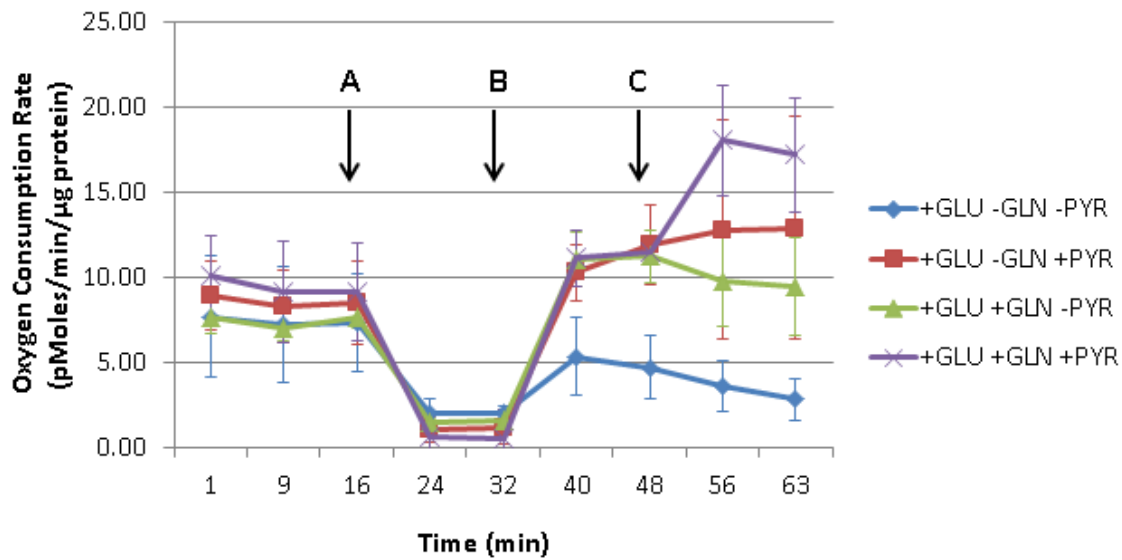
for the production of ATP. Consequently, the metabolic balance within the platelet would be shifted away from oxidative phosphorylation towards the glycolytic pathway, resulting in an increase in lactate formation and a decrease in pH. Future studies will be necessary to determine if EDTA is a better suited anticoagulant compared to citrate solutions with reduced glucose concentrations.

### **Energy Substrate Preferences**

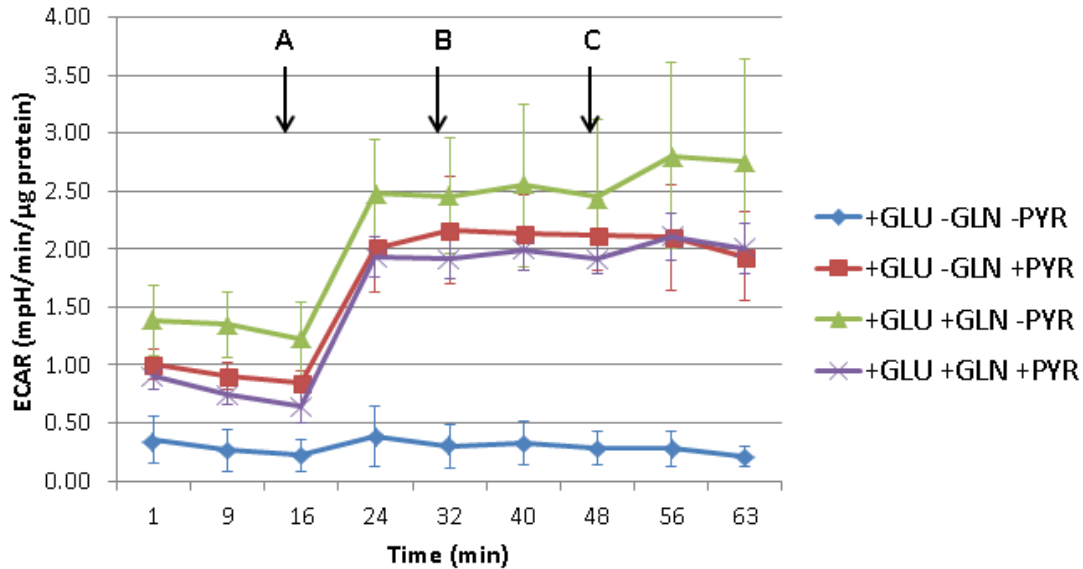
The running buffer used for analysis of bioenergetic function was supplemented with physiologically relevant concentrations of glucose, glutamine, and pyruvate. At their basal state, platelets actively consume glucose and other metabolic fuels resulting in an oxygen consumption rate of approximately 200-250 (pMoles/min per  $6 \times 10^7$  platelets) (Refer to Figure 11). To account for platelet seeding efficiencies, oxygen consumption rate was normalized to  $\mu\text{g}$  of protein; basal oxygen consumption rates in the presence of 5 mM glucose range from approximately 7-9 (pMoles/min/ $\mu\text{g}$  protein) (Refer to Figure 12). The basal extracellular acidification rate of platelets in the presence of glucose ranged from 0.22-1.22 (mpH/min/ $\mu\text{g}$  protein), indicating that a limited proportion of glucose metabolism occurs through glycolysis resulting in lactate acid formation (Refer to Figure 13). The addition of 2.5 $\mu\text{M}$  oligomycin allowed for the assessment of oxygen consumption rate linked to ATP production. When normalized to  $\mu\text{g}$  of protein per well, 81% of oxygen consumption by platelets isolated from a healthy donor is dedicated to ATP production. Taken together, these results confirm that in their basal energy state, platelets primarily metabolize glucose through oxidative phosphorylation rather than glycolysis.



**Figure 11: Oxygen consumption rate (pMoles/min) per  $6 \times 10^7$  platelets in the presence of glucose, glutamine, and or pyruvate.** Platelets isolated from a healthy donor were suspended in media containing 5 mM glucose, 4 mM glutamine, and or 1 mM pyruvate. After three baseline measurements of oxygen consumption rate, respiration was inhibited by the addition of  $2.5 \mu\text{M}$  oligomycin (A). Two subsequent additions of  $15 \mu\text{M}$  of 2,4 DNP were added to stimulate maximum respiration (B, C). Each data point is the average of 3-4 independent wells.



**Figure 12: Oxygen Consumption Rate (pMoles/min/ $\mu$ g protein) in the presence of glucose, glutamine, and or pyruvate.** Platelets isolated from a healthy donor were suspended in media containing 5 mM glucose, 4 mM glutamine, and or 1 mM pyruvate. Measurements were normalized to  $\mu$ g of protein per well. After three baseline measurements of oxygen consumption rate, respiration was inhibited by the addition of 2.5  $\mu$ M oligomycin (A). Two subsequent additions of 15  $\mu$ M of 2,4 DNP were added to stimulate maximum respiration (B, C). Each data point is the average of 3-4 independent wells.



**Figure 13: Extracellular Acidification Rate (mpH/min/ $\mu\text{g}$  protein) in the presence of glucose, glutamine, and or pyruvate.**

Platelets isolated from a healthy donor were suspended in media containing 5 mM Glucose, 4 mM Glutamine, and or 1 mM Pyruvate. Measurements were normalized to  $\mu\text{g}$  of protein per well. After three baseline measurements, proton production was stimulated by the addition of 2.5  $\mu\text{M}$  Oligomycin (A). Two subsequent additions of 15  $\mu\text{M}$  of 2,4 DNP were added (B, C). Each data point is the average of 3-4 independent wells.

In addition to glucose, the running buffer was also supplemented with 4 mM glutamine and 1 mM pyruvate. To assess if the addition of glutamine and or pyruvate increases the bioenergetics capacity of platelets, the spare respiratory capacity was calculated for each condition. The spare respiratory capacity is an indication of a cell's ability to exceed its basal oxygen consumption rate, usually under stress inducing conditions. The addition of glutamine increased the spare respiratory capacity by 106%. The addition of pyruvate increased the spare respiratory capacity by 180%. Together, the addition of glutamine and pyruvate synergistically increased the spare respiratory capacity by 221% (Refer to Table 2). In addition to spare capacity, respiratory control ratio is frequently used as a measurement of healthy mitochondrial activity. The

respiratory control ratio is defined as the maximum respiration stimulated by a proton ionophore divided by the minimum oxygen consumption rate induced by the addition of an ATP synthase inhibitor. A high RCR indicates that the mitochondria have a high capacity for substrate oxidation and ATP turnover, and low proton leak. Platelets suspended in media containing pyruvate had higher respiratory control ratios compared to platelets supplemented in similar media without pyruvate. Compared to platelets supplemented with only glucose, platelets supplemented with glucose and pyruvate exhibited a 335% increase in their RCR. When platelets are supplemented with both glucose and glutamine, the addition of pyruvate increases the RCR by 281%. The increased spare respiratory capacity and heightened respiratory control ratios suggest that platelets are able to use exogenous sources of energy, including glutamine and pyruvate. The addition of these substrates increases the platelets ability to generate ATP and their tolerance to stressful environments. These results suggest that alternative biofuels, e.g. pyruvate, could be used to maximum platelet bioenergetics during *in vitro* storage.

	<b>Spare Respiratory Capacity</b>	<b>Change in Spare Respiratory Capacity (% of Control)</b>	<b>Respiratory Control Ratio</b>	<b>Change in Respiratory Control Ratio (% of control)</b>
Glucose + Glutamine	0.17	106%	4.09	121%
Glucose + Pyruvate	2.29	180%	8.06	335%
Glucose + Glutamine + Pyruvate	3.45	221%	15.59	281%

**Table 2: Change in Spare Respiratory Capacity and Respiratory Control Ratio**

Platelets isolated from a healthy donor were suspended in media containing 5 mM Glucose, 4 mM Glutamine, and/or 1 mM Pyruvate (n=2). Measurements were normalized to  $\mu\text{g}$  of protein per well. Spare respiratory capacity and respiratory control ratio were calculated for each condition. Change in spare respiratory capacity and respiratory control ratio are reported as the percent increase compared to the control (glucose alone).

### **Comparison of Platelet Bioenergetics Pre and Post Cardiopulmonary Bypass Surgery**

Previous studies have demonstrated that cardiopulmonary bypass activates platelets resulting in structural and biochemical changes (19). The ramifications of cardiopulmonary bypass on platelet bioenergetics have not yet been evaluated. We hypothesized that changes in platelet-dependent blood coagulation after bypass are the result of impaired platelet energy metabolism. To evaluate platelet energy metabolism in cardiopulmonary bypass patients, samples of whole blood were obtained immediately before and following surgery. Platelets were isolated from whole blood samples and plated at  $3 \times 10^7$  or  $6 \times 10^7$  depending on the patient's total platelet count. Platelet seeding was performed using two different techniques. The first four patients' platelets were seeded using a 140g centrifugation for 10 minutes. All subsequent patients' (n=6)

platelets were seeded using the previously optimized centrifugation technique, 1600g for 5 minutes with no brake; the plate was then rotated 180° and centrifuged for an additional 5 minutes at the same speed.

Before optimization of the platelet seeding technique, the average maximum oxygen consumption rate (OCR) was  $6.75 \pm 4.99$  (pMoles/min/ $\mu$ g protein). After surgery the average maximum OCR drops to  $2.5 \pm 0.56$  (pMoles/min/ $\mu$ g protein). The standard deviation is more than 50% of the average OCR for both pre and post-surgery; the wide margin of measured values could be an artifact of patient variability, but alternatively it could be indicative of inadequate seeding technique prior to the assay. The average respiratory control ratio (RCR) for these patients was 4.83 before bypass and 1.00 after. Implementation of the new centrifugation technique during seeding yielded higher maximum OCRs in patients both before and after bypass,  $11.00 \pm 2.9$  (pMoles/min/ $\mu$ g protein) and  $9.92 \pm 2.02$ , respectively (n=6). The average respiratory control ratio also increased in patients both before and after bypass. Results obtained with the newly optimized platelet seeding technique suggest that cardiopulmonary bypass does not affect mitochondrial bioenergetics in patients.

Centrifugation Technique	Baseline (pMoles/min/ $\mu$ g protein)		Oligomycin-induced State 4 Respiration (pMoles/min/ $\mu$ g protein)		DNP-induced State 3 Respiration (pMoles/min/ $\mu$ g protein)		Respiratory Control Ratio		Spare Respiratory Reserve Capacity	
		Std Error		Std Error		Std Error		Std Error		Std Error
OLD PRE	5.88	2.69	1.25	0.41	6.75	4.99	4.83	2.95	5.50	4.78
OLD POST	2.38	0.54	0.50	0.43	2.50	0.56	1.00	0.00	2.00	0.79
NEW PRE	5.22	1.02	1.67	0.12	11.00	2.87	5.67	1.35	5.40	1.50
NEW POST	4.86	0.73	1.67	0.07	9.92	1.88	6.15	1.17	4.62	2.05

**Table 3: Bioenergetic Profile for Cardiopulmonary Bypass Patients**

Platelets isolated from whole blood samples obtained from cardiopulmonary bypass patients immediately before and following surgery. Measurements were normalized to  $\mu$ g of protein per well. N = 4 for the "old" centrifugation and n = 6 for "new" centrifugation technique. The optimized centrifugation technique used during platelet seeding increased the average maximum oxygen consumption rate and average respiratory control ratio in patients both before and after bypass.

### Bioenergetic Profile for Stroke Patients Suffering from Depression-like Symptoms

Platelets were isolated from whole blood samples from patients who have recently suffered a stroke and presently experience depression-like symptoms and fatigue. Comparing the two patients to a healthy control did not reveal an obvious trend in altered platelet bioenergetics (Refer to Table 4). Compared to the healthy control, PT1001 had a similar basal OCR and oligomycin-induced state 4 respiration. However, PT1001 had a 24% decrease in DNP-stimulated state 3 respiration; ultimately resulting in a 25% decrease in respiratory control ratio and a 68% decrease in respiratory reserve capacity compared to the healthy control. These preliminary results suggest that post-stroke patients suffering from fatigue and depression-like symptoms have reduced platelet functionality. Whether the reduced platelet functionality is a cause or consequence of stroke remains to be elucidated.

Conversely, PT1005 had a 150% increase in basal OCR compared to the healthy control. State 4 and state 3 respirations were also elevated above the control, ultimately contributing to a 5% increase in the respiratory control ratio compared to the control. A 108% increase in reserve capacity compared to the control was observed. These results suggest that the platelets from this patient were hyperactive; a finding consistent with previous reports suggesting that hyperfunctional platelets may contribute to the development of an acute thrombotic event and are

a cause rather than a consequence of stroke (45). Additional patients are necessary to determine which bioenergetic profile is more common among post-stroke patients suffering from fatigue and depression-like symptoms. In addition, the healthy controls used from comparison should be age and sex-matched to reduce any variations resulting from differences in age or gender.

	<b>Baseline OCR ± Std Error (pMoles/min/μg protein)</b>	<b>State 4 Respiration ± Std Error (pMoles/min/μg protein)</b>	<b>State 3 Respiration ± Std Error (pMoles/min/μg protein)</b>	<b>Respiratory Control Ratio</b>	<b>Respiratory Reserve Capacity</b>
PT1001	4.91 ± 0.32	1.22 ± 0.14	6.15 ± 0.53	5.03	1.24
PT1005	10.62 ± 1.49	2.62 ± 0.72	18.61 ± 5.26	7.11	7.99
Healthy Control	4.23 ± 0.16	1.22 ± 0.06	8.07 ± 0.83	6.74	3.84

**Table 4: Bioenergetic Profile for Stroke Patients Suffering from Fatigue and Depression-like Symptoms**

Platelets were isolated from whole blood samples from patients who have recently suffered a stroke and presently experience fatigue and depression-like symptoms. All values reported are the average of three independent wells and are normalized to μg of protein per well. State 4 respiration induced with 2.5 μM Oligomycin. State 3 respiration stimulated by two sequential additions of 15 μM 2,4-DNP.

### Storage of Platelet Concentrates

Platelet concentrates were obtained from Red Cross Holland Labs. Bioenergetic measurements were obtained on Days 0, 3, and 6 (Refer to Table 5). Platelets were seeded at a concentration of  $6 \times 10^7$  per well. On day 0, the average basal OCR normalized to μg protein was 1.01 (pMoles/min/μg protein); significantly less than the respiratory control ratio of platelets obtained from the healthy control used in previous studies, 4.93 (pMoles/min/μg protein). Annexin V staining of these platelets on Day 0 revealed that 16.8% of total cells had undergone apoptosis. The level of apoptosis supports the low average basal OCR. On day 6, the average basal OCR normalized to μg protein decreased to 0.59 (pMoles/min/μg protein) and was accompanied by an increase in annexin V positive cells, 30.2%. These results indicate that as platelet storage time increases, the percent of platelets undergoing apoptosis increases. Additionally, the platelets that do survive to later time points exhibit decreased capacity to respire. Future

studies will assess the bioenergetic profile of freshly isolated platelets obtained from healthy donors and stored over a nine day time course.

	<b>Mean Basal OCR (pMoles/min/<math>\mu</math>g protein)</b>	<b>Percent of Platelets Positive for Annexin V</b>
Day 0	1.01 $\pm$ 0.89	16.8
Day 3	1.61 $\pm$ 0.66	23.6
Day 6	0.59 $\pm$ 0.09	31.8

**Table 5: Average Basal Oxygen Consumption Rate and Percent Positive Annexin V in Platelet Concentrates**

Mean basal OCR was measured in platelet concentrates over a six day timecourse. Results reported are the average of three triplicate wells. Measurements were normalized to  $\mu$ g of protein per well. Results presented are the average of triplicate wells in a single assay. As the mean basal OCR decreases, the percent of platelets staining positively for annexin V.

## **Limitations & Key Assumptions**

All platelets used in the studies described here were derived from a single donor. While this helps to eliminate inconsistencies during optimization assays due to donor variability, it also assumes that the single donor used represents the population. Using multiple donors and pooling platelets for measurements would potentially eliminate variability as a result of donor differences. In essence, pooling from multiple donors would allow for the establishment of an average bioenergetic profile.

The bioenergetic profile of platelets suspended in media other than plasma could alter the bioenergetic profile. The current platelet storage media approved by the United States Airforce consists of 34 mM Citric acid and 74 mM Sodium Citrate as anticoagulants, and 123 mM Glucose. The running buffered used for Seahorse measurements, recommended by Seahorse Biosciences, has physiological levels of energy substrates including 5 mM Glucose, 4 mM Glutamine, and 1 mM Pyruvate. Both the Seahorse running buffer and the platelet storage media approved by the United States Airforce lack chemokines and other mediators normally found in plasma. Future studies will be necessary to evaluate platelet mitochondrial respiration in plasma compared to the Seahorse running buffer and platelet storage media.

## **Future Directions**

Several studies have published methods for isolating and defining distinct bioenergetic profiles in blood cells, including platelets, in hopes of using blood cells as a diagnostic tool to assess the role of mitochondrial dysfunction in pathologies (15, 16, 22, 39). Using the same methodologies we were unable to produce a reliable and reproducible bioenergetic profile for platelets. Using an optimized isolation technique we were able to reliably isolate platelets from whole blood with a greater than 70% yield. Suspending platelets in a lightly buffered medium supplemented with physiological levels of energy substrates facilitated normal energy metabolism in platelets at their basal state. Seeding at  $6.0 \times 10^7$  platelets per well using a 1600g centrifugation spin allowed for adherence without activation. These techniques culminated in the establishment of a reliable and reproducible bioenergetic profile for platelets at their basal energy level.

Establishing a reliable and reproducible basal bioenergetic profile for platelets is a critical component of achieving our principal objective. It will allow us to compare different storage mediums and to assess mitochondrial dysfunction in different patient populations. After establishing a reliable and reproducible method to assess bioenergetic function in intact platelets, we will evaluate the effectiveness of different alternative biofuels, antioxidants, and other mitochondrial drugs in platelet storage solutions to extend the lifespan of stored platelet concentrates. We hypothesize that platelet storage time is limited by mitochondrial energy metabolism and that the inclusion of alternative biofuels, antioxidants, and other mitochondrial drugs will attenuate mitochondrial dysfunction and thereby increase platelet storage time. We will also work to elucidate the

relationship between platelet oxidative phosphorylation, anaerobic glycolysis, ATP levels, and oxidative stress during extended storage times.

Additional cardiopulmonary bypass patients and post-stroke patients suffering from fatigue and depression-like symptoms are necessary to elucidate alterations in platelet mitochondrial bioenergetic profiles as a result of these pathologies. Age and sex-matched controls will be recruited for comparisons to eliminate any variations due to age or gender.

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