POLITECNICO DI MILANO

Research Doctorate Course in Bioengineering

XXVI Cycle

Final dissertation

Multi-perspective investigation of the effectiveness of anti-thrombotic treatments in association with shear-mediated platelet activation

Lorenzo VALERIO

Advisors:
Prof. Alberto Redaelli
Prof. Gianfranco B. Fiore
Prof. Marvin J. Slepian

Coordinator of the Research Doctorate Course
Prof. Andrea Aliverti

Tutor
Prof. Maria Cristina Tanzi

11 December 2014

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Papers on international peer-reviewed journals (relevance to dissertation)

1. Valerio L.*, Tran P.L., Sheriff J.*, Brengle W., Ghosh R., Chiu W.C., Redaelli A., Fiore G.B., Pappalardo F., Bluestein D., Slepian M.J. “Aspirin as a Means of Modulating Shear-Mediated Platelet Activation Over a Range of Shear Stress”. Journal of Thrombosis and Thrombolysis. *shared 1st author. Under submission.

2. Valerio L., Brengle W., Tran P.L., Hutchinson M., Chiu W.C., Sheriff J., Redaelli A., Fiore G.B., Pappalardo F., Bluestein D., Slepian M.J. “Do Current Anti-Platelet Agents Truly Provide Protection Against Shear-Mediated Platelet Activation in Mechanical Circulatory Support?”. JHLT. Under submission.

3. Tran P.L.*, Valerio L.*, Yamaguchi J.T., , Brengle W., Sen N., DeCook T., Redaelli A., Slepian M.J. “Desensitization of DMSO-treated Platelets to Non-Physiological Shear Stresses and Common Agonists via Membrane Modulation”. Thrombosis Research *shared 1st author. In preparation.

Other papers on international peer-reviewed journals

1. Leopaldi A.M., Vismara R., Lemma M., Valerio L., Mangini A., Contino M., Redaelli A., Antona C. and Fiore G.B., “In vitro hemodynamics and valve imaging in passive beating hearts”. Journal of Biomechanics, 2012, 45(7):1133-39.

2. Tran P.L., Valerio L., DeCook T., Hutchinson M., Brengle W., Sheriff J., Bluestein D., Slepian M.J. “Platelet Activity State in Human, Bovine, and Ovine Under Non-Physiological Shear Stress: a Comparative Study”. ASAIO. In Preparation.

3. Tran P.L., Sen N., Valerio L., Momayez M., Bluestein D., Parthasarathy S., Slepian M.J. “Acoustic-Mediated Platelet Activation: A Novel Pro-Thrombotic Mechanism Linking Obstructive Sleep Apnea and Cardiovascular Disease”. Science Translational Medicine. In Preparation.

Conference presentations

1. Tran P.L., Valerio L. et al. How You "Place It" Matters: The Impact of Inflow and Outflow VAD Cannulation Orientation on Overall System Thrombogenicity. Seventh Annual Frontiers in Biomedical Research Poster Forum. Sarver Heart Center, Tucson AZ, 30 October 2013.

2. Valerio L., Tran P.L. et al. Acoustic-Mediated Plaletlet Activation: A Novel Pro-Thrombotic Mechanism Linking Obstructive Sleep Apnea and Cardiovascular Disease. Seventh Annual Frontiers in Biomedical Research Poster Forum. Sarver Heart Center, Tucson AZ, 30 October 2013.

3. Valerio L., Tran P.L., Brengle W., Hutchinson M., Sheriff J., Bluestein D., Slepian M.J. Do Current Anti-Platelet Agents Truly Provide Protection Against Shear-Mediated Platelet Activation in Mechanical Circulatory Support? ISHLT; 34th Annual Meeting and Scientific Session, San Diego, 10-13 April 2014.

4. Sheriff J., Tran P.L., Valerio L., Ghosh R., Brengle W., Zhang E., Hutchinson M., Bluestein D., Slepian M.J. Antiplatelet Drug Efficacy Under Dynamic Device-Related Shear Conditions. 2014 BMES Annual Meeting. San Antonio, 22-25 October 2014.

Conference proceedings

1. Leopaldi A.M., Valerio L., Lemma M., Vismara R., Cervo M., Mangini A., Contino M., Fiore G. B., Antona C., Redaelli A., “A New Pulsatile Mock Loop For In Vitro Simulation Of Heart Valve Procedures In Porcine Heart”. Abstract, 4th Joint ESAO-IFAO Congress 2011, Porto, 9-12 October 2011.

2. Tran P.L., Valerio L., Brengle W., DeCook T., Hutchinson M., Khalpey Z., Poston R., Chiu W-C., Merkle V., Sheriff J., Bluestein D., Slepian M.J. Device Thrombogenicity Emulation (DTE)-Assisted VAD Design Improvement is associated with Reduced Platelet Activation In Vivo. Oral Presentation. 21st Congress ISRBP, Yokohama (Japan), 26 – 28 September 2013.

3. Tran P.L., Valerio L., Brengle W., Poston R., Slepian M.J.. Robotic Implantation of VADs: The Next Step Forward. Oral Presentation. 21st Congress ISRBP, Yokohama (Japan), 26 – 28 September 2013.

4. Tran P.L., Valerio L., Yamaguchi J., Brengle W., DeCook T., Hutchinson M., Sen N., Bluestein D., Slepian M.J. Dimethyl Sulfoxide: a new nemesis of shear-induced platelet activation. ASME 2014 3rd Global Congress on Nano-Engineering for Medicine and Biology (NEMB 2014), San Francisco CA, 2-5 February 2014.

5. Valerio L., Tran PL, Brengle W., Sheriff J., Redaelli A., Bluestein D., Slepian MJ. Do Current Anti-Platelet Agents Truly Provide Protection Against Shear-Mediated Platelet Activation in Ventricular Assist Devices? Oral Presentation. 22st Congress ISRBP, San Francisco (USA), 25 – 27 September 2014.

Abstract

Ventricular assist devices (VADs), the most prominent solution for the treatment of heart failure (HF), are still burdened with several post-implant complications, such as pump failure, infections or thrombotic events. Among these, the latter are largely due to the interaction between VADs and blood, requiring a challenging integration of technical, clinical and biological expertises to be solved. Increased shear stresses are a hallmark of the flow conditions in blood recirculating devices, and patients implanted with such devices require lifelong anti-thrombotic therapies to counteract the high risk of thromboembolism.

Although antiplatelet agents have proven their effectiveness as biochemical inhibitors of platelet activation, their behavior under shear stress, i.e. in response to physical forces encountered when the blood flows through VADs, has been only marginally investigated.

In the present dissertation, cutting-edge bioengineering techniques are employed to investigate the ability of pharmacological treatments to reduce VADs thrombogenicity after exposure to complex dynamic shear stress profiles. Our studies involve a methodology, developed in the last decade, which integrate complex numerical and experimental approaches *in silico* and *in vitro*, allowing a comprehensive investigation of device-related platelet activation. Platelet activity state in such conditions was monitored using a specific prothrombinase assay, the PAS assay. Different antiplatelet drugs that are commonly administered to VADs patients were tested, both individually and in combination, and quantitatively compared. Results suggest that such drugs are only partially able to protect platelets from the activation effects exerted by physical forces acting within cardiac assist devices.

New mechanisms of action were also studied as possible future solutions to overcome the limitations associated with current therapies. Our studies indicate that a paradigm shift is required in the development of new antiplatelet drugs for the treatment of shear-mediated platelet activation. In particular, the discovery of new agents able to affect platelet membrane fluidity might reduce the need for high-impact antithrombotic therapies, offering an effective protection to platelets when exposed to high shear stress conditions.

1

Introduction

1.1 Background

Heart Failure (HF) is having a growing diffusion in the world and can be considered one of the most serious public health burden of the 21st century. It affects nearly 5.8 million people in the United States and over 23 million worldwide (Liu et al., 2014). HF is accompanied by increasing clinical costs and a need for devices able to restore the correct function of the heart. So far, heart transplant represent the most prominent solution for the treatment of HF (Roger et al., 2012). However, the paucity of heart donors compared to the large need of heart replacement, have brought to the development of new blood recirculating devices. The latter include mechanical circulatory support (MCS) implants, such as ventricular assist devices (VADs) and total artificial hearts (TAHs) (MacIntyre et al., 2000; Konstam MA, 2000).

The implementation of MCS devices nowadays represents a prominent technological solution for the treatment of HF, requiring a challenging integration of technical, clinical and biological expertises. In this scenario, bioengineers play a key role in the attempt of matching the requirement of clinicians, who call for new and better performing devices able to allow the treatment of HF, and the economic dynamics, which still register extremely high costs for the development or the optimization of such devices. Despite enormous investment of both human and financial resources for the realization of MCS devices, the latter are still burdened with several complications such as recurrent pump thrombosis, stroke or thromboembolic events, which still have been only marginally studied (Slaughter et al., 2010; Gregoric ID, 2012; Kirklin et al., 2013). These adverse events are largely due to the interaction between these devices and the blood. This aspect is often treated with a mostly empirical approach by the MCS manufacturers, who use different methodologies for the characterization of the technological performances of the devices (CFD simulations) or the investigation of their effect on the biological environment in which they are meant to operate (*in vitro* campaign, animal models). It appears clear that a comprehensive approach should rather be pursued, involving a combination of technical and biological efforts interacting with each other to give birth to better devices, capable of restoring the normal blood flow in the body without having an excessive impact on the biological structures.

In order to achieve this goal, in the first phase of development of new devices, CFD simulations of the interaction between blood constituents, flow fields, and blood-contacting surfaces should be performed to characterize the devices thrombogenicity (Bluestein et al., 2010; Goodman et al., 2005; Xenos et al., 2010). Moreover, in an ideal methodological setting, such computational approaches should be integrated with methods able to reproduce

in vitro the fluid dynamic conditions encountered *in vivo*. The need to investigate the thrombogenicity of cardiac assist devices using this combined approach has led to the creation of the Device Thrombogenicity Emulator (DTE), a method developed by Stony Brook University (Prof. Danny Bluestein) in collaboration with the University of Arizona (Prof. Marvin J. Slepian). This technology facilitates optimization of devices performing first *in silico* analysis in the modeling domain (in which virtual design modifications are examined), followed by experimental emulation of the device specific stress loading histories (waveforms) *in vitro* using the hemodynamic shearing device (HSD). The latter consists of a computer controlled cone-and-plate viscometer with the capability of exposing platelets to highly-controlled dynamic shear stress patterns (Girdhar et al., 2008). The DTE, coupled with the platelet activity state (PAS) assay (Jesty et al., 1999) that allows to determine a one-to-one relation between thrombin formation and platelet activity state, became an extremely robust tool to assess the thrombogenicity of different cardiac devices. Several studies combining experimental and predictive approaches have been conducted based on the DTE method (Nobili et al., 2008; Sheriff J, Bluestein D et al., 2010; Sheriff et al., 2013; Girdhar et al., 2012).

This methodology can be also used to assess the ability of antiplatelet agents to inhibit platelet activation after exposure to shear stress profiles, such as the ones encountered within VADs. Up to now, only a few studies in the literature focused on this research topic (Moake et al., 1988; Sheriff et al., 2014; Minami et al., 1997; Tomizawa et al., 2013).

This approach would allow to define new strategies for the inhibition of shear-mediated platelet activation, paving the road towards the development of more effective anti-thrombotic pharmacological agents.

1.2 Aims of the thesis

The DTE methodology allows investigating the effect of several chemical agents on platelets using a platform (HSD) able to reproduce with high fidelity the shear stress profiles that blood encounters within VADs. With this approach, short and long term aims could be achieved with the goal of discovering new feasible solutions to reduce the burden of antithrombotic therapies, that are up to now essential for a safe clinical use of MCS devices.
In the present thesis a detailed investigation is performed to assess the ability of traditional and unconventional antiplatelet treatments to protect platelet from shear-mediated activation. In this context, one of the short term aims is to test the major antiplatelet agents nowadays on the market, thus allowing to determine whether or not they are able to provide protection to platelets exposed to shear stress profiles. Moreover, we study the ability of an unconventional chemical agent, the dimethyl sulfoxide (DMSO), to offer a protection from shear-mediated activation by acting on platelet membrane. This effect may offer a paradigm shift in the development of new drugs for the treatment of platelet activation.

The long term aim of this study is thus to determine the major triggers involved in shear-mediated platelet activation. This discovery would lead to define better therapies able to face the burden of massive antithrombotic regimens associated with MCS implants.

Finally, to extend the possible clinical use of PAS assay in detecting platelet activation state, we performed a set of pilot studies involving its use both *in vitro* with whole blood or purified platelets and *in vivo* with animal models.

The aims described above will be pursued by the implementation of specific goals achieved using a variety of experimental tools. These specific aims are broken down as follows:

Aim 1. To determine the degree of platelet activation experienced by platelets exposed to defined high (“hotspot”) and low dynamic shear stress waveforms extracted from CFD simulations within the DeBakey VAD.

Aim 2. To examine the ability of current anti-platelet agents to limit the activation of platelets exposed to both constant and dynamic stress loads derived from the shear profiles of the DeBakey VAD.

Aim 3. To verify the capacity of DMSO to inhibit platelet activation, an effect that we hypothesized owing the DMSO's property of enhancing membrane fluidity, thus allowing platelets to better face shear stress profiles as the ones encountered within VADs.

Aim 4. To validate the PAS assay as method to detect platelet activation, in order to use the assay in further diagnostic devices able to monitor platelet activity state of MCS patients.

1.3 Outline of the thesis

The thesis is focused on three main aspects and is structured as follows:

Chapter 2 describes the state of the art regarding heart failure treatments. Moreover, MCS devices and the historical evolution of methods for the assessment of their thrombogenicity will be discussed, as well as the major mechanisms of action that characterize antiplatelet therapies.

In Chapter 3, the ability of several antiplatelet agents to provide a protective effect over shear-mediated platelet activation is investigated. Purified platelets pre-incubated with drugs are exposed to both constant and dynamic shear stress profiles via HSD. The effect of drugs on platelet activation is monitored using a chemical assay already described in literature, the PAS Assay (Jesty et al., 1999).

Chapter 4 analyzes the effect of different concentration of DMSO on shear-mediated platelet activation. The ability of DMSO to increase platelet membrane fluidity and to protect platelets from shear stress exposure is investigated using both the HSD and the syringe-capillary shearing device (SCSD). The latter consists in a pc-controlled syringe pump coupled to a series of capillaries with defined geometries able to subject platelets to hyper shear stress conditions (> 70 dyne/cm^2), not achievable with the HSD. Even during these experiments, the platelet activity state of samples pre-treated with DMSO and exposed to shear is assessed using the PAS assay.

Chapter 5 reports two pilot studies conducted to validate the PAS assay as possible diagnostic method for platelet activation related to MCS devices.

The first study consists of an *in vitro* investigation of two VADs (Debakey VAD and HeartAssist 5) in appropriate bench loops, using both gel-filtered platelets (GFP) and whole blood (WB) as experimental fluids.
HA5 is further investigated with an *in vivo* campaign on calves, where the VAD is implanted and its effect on platelet activation monitored using PAS assay for a total of 2 hours after the implant.

In the last Chapter, a general discussion of the PhD dissertation and the conclusive remarks are presented.

The work described in this dissertation arises from an intense collaboration between the Biomechanics Group of Politecnico di Milano (Milan, Italy) and the Bioengineering Group of the University of Arizona (Tucson, Arizona).

2

Background

2.1 Heart failure: pathophysiology, treatments and the role of mechanical circulatory support (MCS) devices

Heart failure (HF) is typically a chronic disease, generally characterized by a progressive deterioration occurring over a period of years or even decades. Due to its increasing incidence, nowadays HF is a large public health burden. It affects nearly 5.8 million people in the United States and over 23 million worldwide, thereby representing a new epidemic of cardiovascular disease (Liu et al., 2014). When HF occurs, the heart cannot pump enough blood to meet the body's needs. It can be the case either that the heart cannot fill with enough blood, that it does not have adequate force to pump blood to the rest of the body, or both of them. A schematic description of HF pathophysiology is described in Table 2.1 (Dickstein et al, 2008).

The leading causes of heart failure are diseases such as coronary heart disease (CHD), high blood pressure and diabetes.

Heart failure is a clinical syndrome in which patients have the following features:
• **Symptoms of heart failure** (breathlessness at rest or on exercise, fatigue, tiredness, ankle swelling) **and** • **Signs of heart failure** (tachycardia, tachypnoea, pulmonary rales, pleural effusion, raised jugular venous pressure, peripheral oedema, hepatomegaly) **and** • **Objective evidence of a structural or functional abnormality of the heart at rest** (cardiomegaly, third heart sound, cardiac murmurs, abnormality on the echocardiogram, raised natriuretic peptide concentration)

Table 2. 1. Definition of Heart Failure (Dickstein et al, 2008).

As the heart's pumping action grows weaker, heart failure becomes more severe. This condition can affect one side of the heart only, but the majority of the cases involve both sides. Right-side heart failure occurs if the heart can't pump enough blood to the lungs to pick up oxygen. On the other hand, left-side heart failure happens when the heart can't pump enough oxygen-rich blood to the rest of the body. The most common causes of functional deterioration of the heart are damage or loss of heart muscle, acute or chronic ischemia, increased vascular resistance with hypertension, or the development of a tachyarrhythmia

such as atrial fibrillation (AF) (Kannel MWB, 2000). So far the most common cause of myocardial disease is CHD, being the trigger pathology in almost 70% of patients with HF. Valve disease accounts for 10% and cardiomyopathies for another 10%. The remaining 10% is due to the effect of drugs or nutritional diseases, as well as endocrine dysfunctions. (Dickstein et al, 2008).

The purpose of diagnosing and treating HF is not different from any other medical condition, namely to reduce mortality and morbidity. Due to the dramatic outcomes of this pathology, particular emphasis has been put on this end-point in clinical trials. However, for many patients, the desire to maximize the duration of life is as much important as the ability to lead an independent life, free from excessively unpleasant symptoms and with low re-admission to hospital. Prevention of heart disease, as well as the ability of correctly manage its progression, remains an essential part of management. Figure 2.1 (Dickstein et al, 2008) provides a treatment strategy for the use of drugs and devices in patients with symptomatic HF and systolic dysfunction.

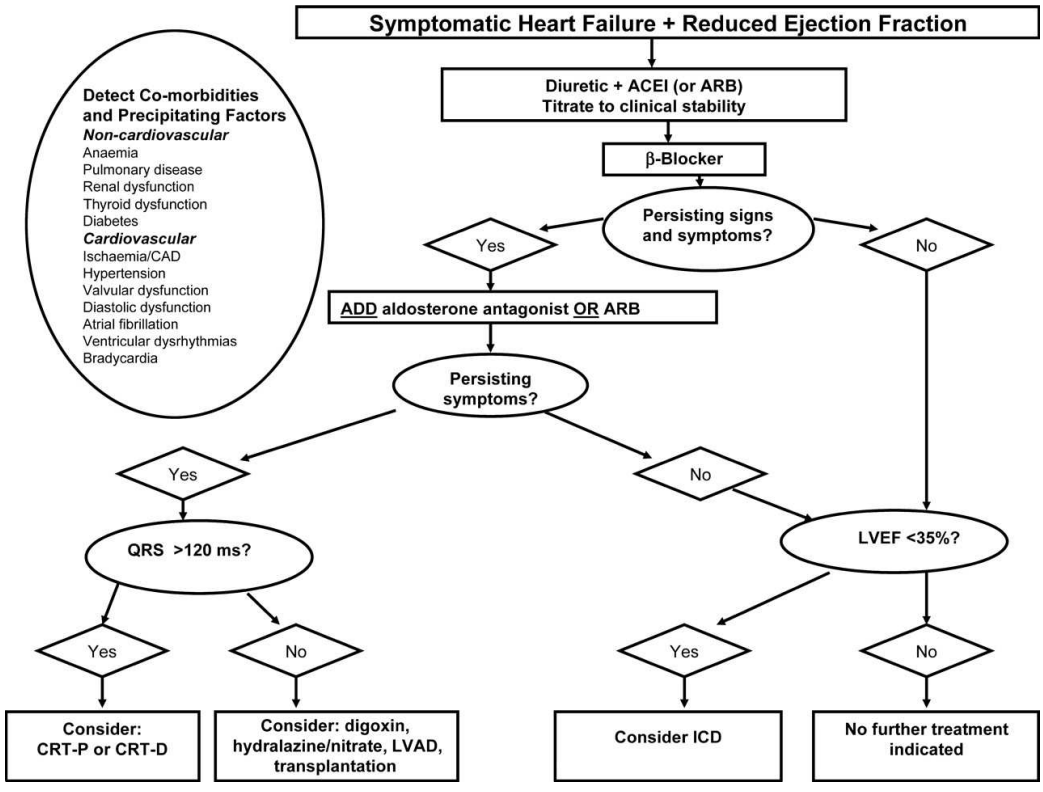

Figure 2.1. A treatment algorithm for patients with symptomatic heart failure and reduced ejection fraction (Dickstein et al., 2008).

In recent years, studies have demonstrated a significant potential for improved clinical outcomes in treatments with medical therapies or mechanical cardiac devices (MacIntyre et al., 2000; Konstam MA, 2000). The possibility of combining drugs and mechanical devices has increased the survival rates and the clinical outcomes of patents previously suffering from severe debilitating medical conditions (Simon et al., 2008).

Pharmacological therapies include drugs that are strictly palliative and can foreshorten life like outpatient intravenous inotropic agents. This therapy should be reserved for those patients who have a reproducible and marked improvement in symptoms after introducing this drugs. Besides pharmacological treatments, heart transplantation remains the definitive solution for advanced and refractory HF. However, heart transplantation remains challenged by inadequate donor supply, finite graft survival, and long-term complications of immunosuppressive therapy. Thus, there is a need for more refined and durable mechanical circulatory support (MCS) options. The recent development of smaller, more durable, and safer ventricular assist devices (VADs) has enabled MCS to emerge as a practical and effective form of therapy, either until heart transplantation can be performed (as bridge to transplantation [BTT]) or increasingly as an alternative to transplantation as Destination Therapy (DT) (Slaughter et al., 2009; Sheikh et al., 2011). In particular, in Figure 2.2 is reported a flow chart developed for helping clinicians to decide which type of device to use based on patient' clinical conditions (Peura et al., 2012).

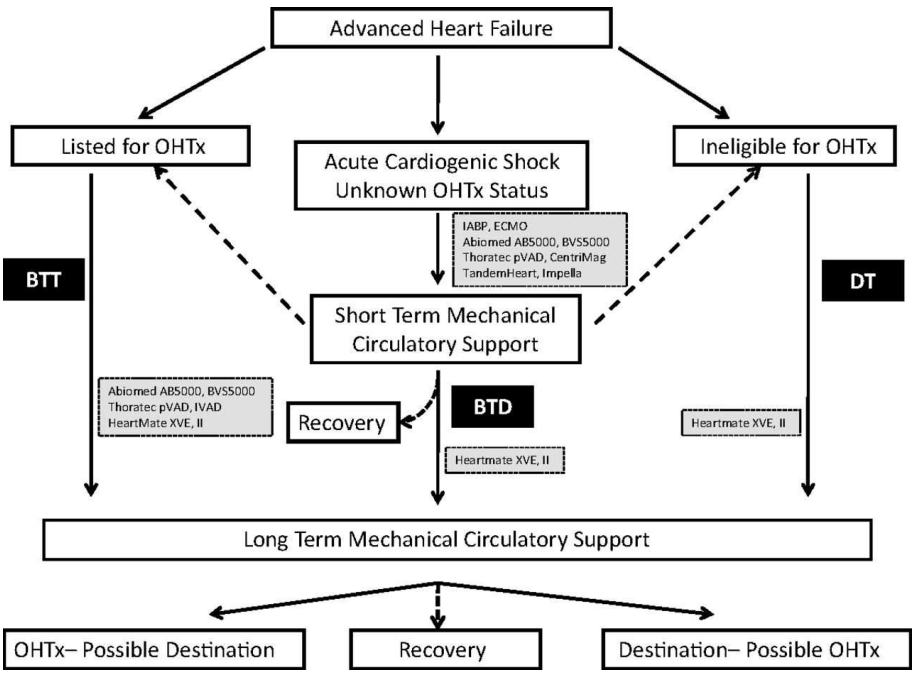

Figure 2.2. Device selection flow chart. OHTx indicates orthotopic heart transplantation; IABP, intraaortic balloon pump; ECMO, extracorporeal membrane oxygenation; pVAD, Paracorporeal Ventricular Assist Device; BTT, bridge to transplantation; DT, destination therapy; and BTD, bridge to decision (Peura et al., 2012).

Among MCS devices, Total Artificial Heart (TAH) was developed during the last 60 years in order to replace the entire cardiac structure in the treatment of severe heart failure that cannot be partially restored with the use of simple VADs. The early development efforts and first reports of successful use of a TAH in animal studies were by Akutsu and Kolff in the 1950s at the Cleveland Clinic (Nosé Y., 2008).

At the University of Utah, Kolff worked with a team on what became the Jarvik-7 TAH; in 1982, the device was first used as permanent destination therapy when DeVries implanted the Artificial Heart in Barney Clark (DeVries et al., 1984), a retired dentist who survived 112 days with the device. Subsequent permanent TAH implants provided support for up to 620 days. To increase the reliability of the Jarvik-7 total artificial heart, The Utah-100 total artificial heart was designed. This device achieved better fit and minimized device associated thrombus formation. The Utah-100 heart was tested by Kinoshita and colleagues in 28 calves and 3 sheep. The smallest animal at the time of implantation weighted 54 kg. Mean survival duration was 78 days (1-331 days), with 14 animals surviving longer than 60 days. Multiple organ function was maintained in normal range and mean plasma free hemoglobin values in the calves that survived longer than 100 days were less than 10 mg/dl.

Despite the ability of the TAH to provide long-term support, the adverse events and compromised quality of life that patients experienced while on device soon redirected the use of the TAH to bridge patients to heart transplant rather than as destination therapy. The first use of the Jarvik-7 model TAH as BTT was performed by Copeland in 1985 (Copeland et al., 1989). A pneumatic TAH developed at Penn State was also implanted shortly afterward in two patients as BTT (Magovern et al., 1986). Since 1985, the Jarvik-7 TAH has been renamed the SynCardia temporary TAH. The latter became the only FDA-approved TAH in the world and has been implanted more than 1100 times (Slepian et al., 2013).

It appears clear as despite their efficacy, MCS devices remain plagued by post-implantation limitations including pump thrombosis, thromboembolic complications and stroke (Slaughter et al., 2010). In fact, continuous anticoagulative therapy is necessary for both surgically and percutaneously placed MCS devices; however, for surgically implanted devices, there is usually a delay before starting the anticoagulation, due to risk of postoperative bleeding. This can result in thrombosis of the device, cardiac chambers, or aorta (Gregoric ID, 2012).

In the 2013 INTERMACS report, 70% of patients implanted with a VAD had experienced some complication within the first year of implantation (Kirklin et al., 2013). Furthermore, Starling et al. outline a rising rate of thrombosis with the HeartMate II (HMII),

with low levels prior to 2011 and a significant increase of devices related failure of the implant from that point to date (Starling et al., 2014).

The high incidence of thromboembolic events in MCS devices occurs largely due to non-physiological flow past constricted geometries within a device, where platelets, the principal cellular clotting elements in blood, are exposed to rapid high shear stress spikes and exposure times (Alemu et al., 2007). Under non-physiological conditions of flow through VADs, platelets are exposed to varying shear stress levels, turbulent stresses, longer residence times in pathological flow regions (“hot-spots”), as well as repeated passages through the device that may precipitate activation, aggregation, and free emboli formation. The investigation of these flow patterns inside the devices results mandatory to evaluate the performances of different cardiac mechanical treatments and to find possible solution to their drawbacks.

2.2 Thrombosis: the Virchow's triad

Thrombosis is the major pathology derived from hemostasis. In thrombosis, a pathological clot, due to abnormal coagulation reactions, uncontrollably enlarges and occludes the lumen of a blood vessel (Kyrle et al., 2009). In 1856, Rudolf Virchow postulated that injury to the vessel wall, hypercoagulability of the blood and abnormalities in blood flow are the three factors that influence thrombus formation (Figure 2.3).

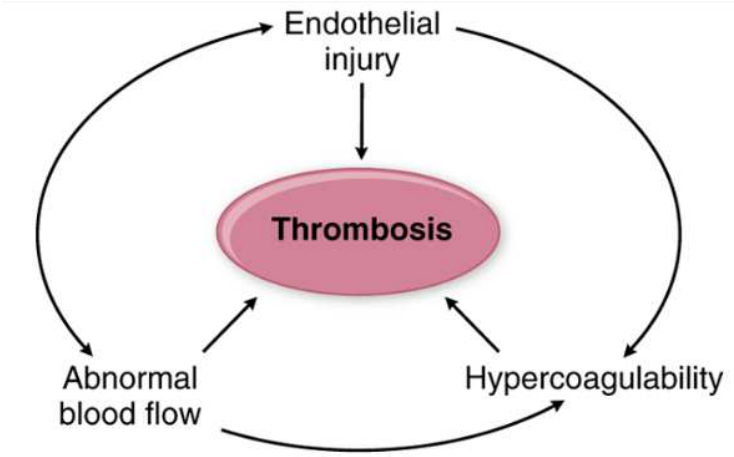

Figure 2.3. The Virchow's triad (Golan et al., 2011).

Endothelial injury is the main cause of thrombus formation in the heart and the arterial circulation (Wu et al., 1996). There are many possible causes of endothelial injury, including changes in shear stress associated with hypertension or turbulent flow, hyperlipidemia, elevated blood glucose in diabetes mellitus, traumatic vascular injury, and some infections (Gaxiola E., 2012). Endothelial injury predisposes the vascular lumen to thrombus formation. This phenomenon can happen through three different phases. First, platelet activators, such as exposed sub-endothelial collagen, promote platelet adhesion to the injured site. Second, the exposure on injured endothelium of thromboplastin, a protein present in subendothelial tissue, initiates the coagulation cascade. Third, natural antithrombotic agents, such as t-PA and PGI_2, become depleted at the site of vascular injury because these mechanisms rely on the functioning of an intact endothelial cell layer (Golan et al., 2011). This lack of antithrombotic agents, combined with platelet activation at the injured site lead to formation of vascular thrombi that eventually generate a severe cardiac pathology.

Hypercoagulability is generally less important than endothelial injury in predisposing to thrombosis, but this condition can be another important factor in some patients. Hypercoagulability refers to an abnormally heightened coagulation response to vascular

injury, resulting from either: primary (genetic) or secondary (acquired) disorders. The genetic form of this disorder affects people born with the pathological tendency to form blood clots. Those hereditary conditions include: Factor V Leiden (the most common), prothrombin gene mutation, deficiencies of natural proteins that prevent clotting (such as antithrombin, protein C and protein S), elevated levels of homocysteine, elevated levels of fibrinogen or dysfunctional fibrinogen (dysfibrinogenemia), elevated levels of factor VIII (still being investigated as an inherited condition) and other factors including factor IX and XI (Wolberg et al., 2012). On the other hand, acquired conditions are usually a result of surgery, trauma, medications or a medical condition that increases the risk of hypercoagulable states. Conditions that would lead to acquired disorders are: cancer, recent trauma or surgery, central venous catheter placement, obesity, pregnancy, supplemental estrogen use, hormone replacement therapy, prolonged bed rest or immobility, heart attack, congestive heart failure, heparin-induced thrombocytopenia (Schafer AI, 1985).

Abnormal blood flow refers to non-physiological fluid dynamic conditions in the vessels. Such conditions include pathological shear stresses that can occur also in presence of laminar flow. Nonetheless, atherosclerotic plaques, as well as bifurcations of blood vessels, can create areas of turbulent flow. Non-physiological flow can also create local pockets where stasis of fluid occurs. Local stasis can result from formation of an aneurysm (a focal out-pouching of a vessel or a cardiac chamber), atrial fibrillation and from myocardial infarction. In the latter condition, a region of non-contractile (infarcted) myocardium serves as a favored site for stasis (Golan et al., 2011). Disruption of physiological blood flow promotes thrombosis by several major mechanisms. In the presence of laminar blood flow and pathological shear stresses, platelets are pushed towards the vessel wall, where they can be activated, promoting thrombi formation (Du et al., 2014). On the contrary, stasis associated with low shear stress inhibits the flow of fresh blood into the vascular bed, so that activated coagulation factors in the region are not removed or diluted. Abnormal blood flow may also promote endothelial cell activation, which leads to a prothrombotic state (Zhao et al., 2007). As described in the previous paragraph, one of the treatment of HF is the use of mechanical circulatory support devices as Intra-Aortic Balloon Pumps (IABP) or VADs. Because of their intrinsic mechanisms of action, these devices create zones of non-physiological flow conditions. Coagulation in the setting of artificial devices involves three areas of interdependence: the blood (platelets, the coagulation cascade, fibrinolysis), the flow over the material, and the blood-contacting material (Kormos et al., 2012). Flow fields and shear stresses in VADs vary dramatically among different types of pumps and even in different

regions within the same pump. High-shear regions in a pump may become problematic as passing platelets are transiently activated by the shear. Moreover, areas of recirculation in a pump may trap platelets, increasing their exposure time to the artificial surface while also increasing the local concentrations of agonists released from previously adhering platelets. Hemocompatibility has always been a goal in pump design, along with efforts to maximize blood flow without clinically significant hemolysis, areas of stasis, turbulent flow, or retrograde flow (Eckman et al., 2012). Nonetheless, the problem of VAD-related thrombosis remains a burden in the HF treatment. Recent studies are trying to overcome the limitations of the actual devices in an attempt to ideally reduce their thrombogenicity and the need of concomitant antithrombotic pharmacotherapy that to date are essential for a safe clinical use of VADs (Girdhar et al., 2012).

2.3 The DTE: a novel methodology to investigate the thrombotic effect of different cardiac devices

The development of tools for predicting thrombus formation *in silico* during the design of medical devices have been helped by the recent advances in computing power and mathematical modeling (Bluestein et al., 2010; Goodman et al., 2005). Complex simulation of interaction between blood constituents, flow fields, and blood-contacting surfaces involves a balanced effort to improve both the accuracy of the numerical simulations and the experimental methods necessary for providing input values and validating outcomes (Xenos et al., 2010). The success of such a tool may greatly reduce the cost of developing a VAD while possibly improving the performance of the device. VAD design typically consists of a trial-and-error methodology to reduce the thrombogenicity of the pump; often many design generations are necessary because of the continuous discovery of unforeseen thrombogenic areas. This upgrade of devices' design needs consecutive round of testing, driving up the cost of the product. Even after multiple design iterations and expensive preclinical animal testing, clinical trials free of thrombotic events are not ensured (Goldstein et al., 2003). As discussed previously, progress in VAD CFD research has helped to elucidate some of the non-physiologic flow fields present in mechanical valves and rotating impellers (Alemu et al., 2010); if these results are combined with experimental determinations of protein and platelet interactions with given materials, it may be possible to define relationship between the flowing blood and the VAD characteristics. In the last decades, researchers from Stony Brook University (Prof. D. Bluestein), in collaboration with the bioengineering group of the University of Arizona (Prof. M.J. Slepian) have developed a thrombogenicity predictive technology (Device Thrombogenicity Emulator, DTE) for optimizing the thrombogenic performance of blood recirculating devices, with the ultimate goal of eliminating the need for life-long anticoagulation therapies. The rationale behind this methodology will be utilized as a tool to design better devices. It offers a paradigm shift, by integrating cutting-edge numerical and experimental approaches *in silico* and *in vitro* (Jesty et al., 2003). The possibility to finally attest the behavior of the results predicted *in vitro* with *in vivo* animal study will better certify the effectiveness of the utilized approach. In recent years some manufacturers started employing CFD to optimize their device designs. Nevertheless, those research efforts are still focused on improved hydrodynamics anticipated to reduce thrombogenicity with no direct correlation to coagulation markers. The DTE methodology bridges this gap by integrating the pertinent hemodynamics with the corresponding thrombogenic markers, facilitating for the

first time an actual optimization of the thrombogenic performance (Girdhar et al., 2012; Bluestein et al., 2010).

The optimization process is performed primarily in the modeling domain, which is interfaced to a universal experimental system (i.e., device independent) that starts from numerical simulations of blood flow in the device and replicates the device hemodynamics with great accuracy. After testing the different configurations, measures regarding the resulting thrombogenic potential *in vitro* before and after iterative design modifications will permit to optimize the thrombogenic performance of updated VADs. Specifically, the DTE method combines stress loading waveforms extracted from the numerical flow modeling, a computer-controlled hemodynamics-emulating device based on a viscometer concept (Hemodynamic Shearing Device, HSD) (Girdhar et al., 2008), and a specific Platelet Activity State (PAS) assay (Jesty et al., 1999) Both the HSD and PAS assay will be described in this and the next chapters.

With this approach it is possible to use this information to design out the “hotspots” predicted in the model, optimizing the pump *in silico* for a fraction of the cost of previous design methods. The further possibility of using a computer-controlled viscometer to reproduce *in vitro* the shearing profiles extracted from the numerical simulation allows to test specific flow conditions, as well as antiplatelet agents in an attempt to systematically investigate their effectiveness. This area of research results vital to limit adverse events after implant of the devices, thus improving outcomes and quality of life for VAD patients, as the technology continues to evolve bringing new solutions for the treatment of HF.

2.4 Characterization of shear-mediated platelet activation

2.4.1 Platelet morphology, production and structure

Resting platelets are discoid enucleate cells and have a smooth, rippled surface with an average diameter of 2 - 4 µm. Normal platelet count is in the range 150000 – 350000 pl/µl and they have a life span of approximately 8–10 days. (George JN, 2000; Bautista et al., 1984). Platelets are derived from the fragmentation of megakaryocytes' cytoplasm, which in turn undergo a process of differentiation from the haemopoietic stem cell in the bone marrow. Platelet production is under the control of humoral agents such as thrombopoietin.

The outermost layer of the platelet (the glycocalyx) is a surface coat made up of glycoproteins (GP) (McEver RP, 1990). This layer plays a very important role in platelet function, including adhesion and aggregation, and thus, contributes to hemostasis. There are several receptors on this glycoprotein layer, which bind to different adhesive agents, aggregating agents, inhibitors and procoagulant factors. These include the selectins (such as GMP 140, or P-selectin), the integrins (such as GP I, GP II, GP III, GP IV, GP V), the immunoglobulins and other receptors, such as those for adenosine diphosphate (ADP), collagen, epinephrine or thrombin (Packham MA, 1994). All these elements assist platelets during the adhesion or aggregation process. In fact, GPIIb-IIIa contributes towards adhesion to fibrin and the binding of fibrinogen that facilitate platelet–platelet interaction, while GPIb is important in the attachment of platelets to von Willebrand Factor (vWF) and the vascular sub-endothelium. Furthermore, glycoprotein GPIa-IIa facilitates adhesion to collagen (Kainoh et al., 1992).

Platelets intracellular organelles in the cytoplasm include alpha (α) granules, electron dense granules, the mitochondria, a dense tubular system, peroxisomes and lysosomes. Although α-granules are not entirely specific to platelets, these molecules store a variety of proteins such as platelet factor 4 (a heparin antagonist), platelet-derived growth factor (PDGF), beta thromboglobulin, fibrinogen, vWF, fibronectin and other clotting factors (White et al., 1980). Some of these molecules are also secreted by the endothelial cells (for example, vWF), or are already present in the plasma (fibrinogen). Platelet factor 4 has anti-heparin activity and its binding to the specific sites on platelets' surface may modulate aggregation and secretion induced by low level of platelet agonists (Kamath et al., 2001). Finally, dense platelet granules contain calcium, serotonin and nucleotides, such as adenosine diphosphate

(ADP). Among all molecules stored in platelet organelles, the ones that can be considered specific to platelets are the beta thromboglobulins.

2.4.2 Platelet and Thromboembolism in MCS devices: from contact with artificial surfaces to thrombus formation

Platelets, the primary cellular clotting elements in blood, are vital to hemostasis and critical to issues related to MCS device hemocompatibility. The latter refers to the interaction of prosthetic material with blood and can be measured in terms of impact on hematologic, inflammatory, or immunologic parameters. Such elements have been observed clinically as significant for endothelial and coagulation systems after VADs implantation (Eckman et al., 2012). The interaction of platelets with the vessel walls and its subsequent contribution to thrombosis is of pivotal importance in the etiology and pathogenesis of peripheral, coronary, cerebrovascular or other vascular diseases (Davì et al., 2007). In the case of contact with artificial surfaces, as within VADs, platelet adhesion and activation are even emphasized. The increasing realization that inappropriate platelet activation plays a prime role in the increasing heart diseases led to several studies regarding platelet activation and the relationship between this phenomenon and thrombus formation (Fitzgerald et al., 1986). At the moment of VADs implant, the first hemostatic reaction, in case of vascular endothelial damage, is vasoconstriction. Thereafter, platelets come into action and the coagulation system is activated. Through disruption of the endothelial layer of the vessel wall, tissue factors are exposed, initiating the coagulation cascade (Savage et al., 1998). In the presence of artificial surfaces, as within VADs, platelets form a thin monolayer over the material which help the release of chemical factors stored in platelets, such as ADP, Ca^{2+}, serotonin and β-TG. This monolayer serves as a base for thrombin generation and platelet aggregation. Such micro aggregates attract more activated platelets by expressing negative charged membrane phospholipids (Nieswandt et al., 2011). This phenomenon speeds up the coagulation cascade which would lead to thrombus formation by increasing Factor Xa activation and converting prothrombin into thrombin (Wu et al., 1994). In fact, adhered platelets undergo shape change, form podocytes, and start to secrete thromboxane A_2 (T_XA_2). Platelet activation promotes alteration of the membrane makeup, with exposure of phosphatidylserine (PS) on the outer leaflet of the membrane, being of particular importance to driving the pro-coagulant reactions. Platelet activation also results in a conformational change in integrin αIIbβ3, which then binds fibrinogen and contributes to the formation of platelet aggregates (Shattil et al., 1985). The

final step in the thrombus formation is represented by the thrombin-mediated conversion of fibrinogen in fibrin, whose polymerized form represents the majority of the clot structure. It is therefore clear that platelet activation results from a cascade of events, which begins with the binding of an agonist to its receptor and ends with platelet shape change, aggregation, and secretion (Mazepa et al., 2013).

2.4.3 Response of platelets to different shear stress conditions: *in vitro* studies

Within MCS devices, the most relevant mechanical agonist to platelet-mediated thrombosis formation is represented by fluid mechanical shear stress. In fact, the latter plays a synergistic role with other agonists in platelet activation and is always present in circulation. In physiological conditions, the shear stresses reached at the vessel wall range from 1 dyne/cm^2 in veins to approximately 30 dyne/cm^2 in large arteries. Higher level of stress is approached in arterioles (60 dyne/cm^2), reaching 380 dyne/cm^2 in stenotic vessels. Even though physiologic low shear stresses (2 – 3 dyne/cm^2) do not significantly activate platelets, higher magnitude as well as longer duration of the shear stress may lead to a detectable level of platelets activation (Ku DN, 1997; Sheriff J, 2010).

Early bio-rheological studies focused on stress effects on measures of platelet function. Subsequent studies instead have elucidated basic mechanisms of platelet activation and have shown that platelets follow very different biochemical pathways at elevated shear stresses compared to a low shear stress environment (Hellums JD, 1994; Kroll et al., 1996).

In 1975, within a research program created with the goal of developing a new artificial heart, Brown and colleagues first reported the direct effect of shear stress on platelets in a cone-plate viscometer (Brown et al., 1975). These experiments were undertaken with the hypothesis that shear stress generated by intracardiac devices leads to mechanical platelet damage. These investigators observed that pathologic levels of shear stress (> 50 dynes/cm^2) applied to platelet-rich plasma (PRP) induced changes in platelet morphology, along with secretion and aggregation. The cause of these changes was although uncertain, as well as lysis observed was minimal at shear stresses below 250 dynes/cm^2.

The rising awareness that platelet adhesive process under flow is tightly regulated by multiple ligand-receptor interactions has encouraged the use of epifluorescence and Scanning Electron Microscopy (SEM) to evaluated the real-time changes in platelet morphology under physiological high shear flow in a perfusion chamber (Kuwahara et al., 2002). Kuwahara and

co-workers tested washed platelets resuspended in HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 3 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 0.35% albumin, and 3.5 mmol/L HEPES, pH 7.2) containing 5 µmol/L of a cytoplasmic marker: calcein acetoxymethyl (AM). The actual working fluid used during the experiments was reconstituted blood, consisting in platelets resuspended in HEPES buffer, washed erythrocytes and $CaCl_2$ (1 mmol/L). The inclusion of red blood cells in the reconstituted blood was due to the fact that they are the main suppliers of phosphatidylserine-exposing membranes needed for coagulation resulting in fibrin formation. The working fluid was thus aspirated through the chamber by a syringe pump at a constant flow rate of 0.285 ml/min, producing a wall shear rate of 1500 s^{-1} at 37°C in a thermostatic air bath. The authors show that platelets exposed to physiological rapid blood flow changes dynamically modify their shape at distinct phases of the adhesive process to complete successful thrombogenesis.

The importance of testing whole blood compared to washed platelet solution has been highlighted by Lu and colleagues. The authors compared the sensitivity of human and bovine platelet to shear stress stimulation using multiple platelet activation markers (Lu et al., 2013). A cone-plate rheometer was used to test fresh whole blood samples at shear stresses up to 400 dynes/cm^2 for 2 min. Platelet markers used to assess the response of the biological samples to shear were platelet counts, platelet surface P-selectin expression and serotonin release into blood plasma. The results indicated that exposure to shear stresses above 200 dynes/cm^2 caused significant changes in all three platelet markers for human blood. In contrast, for bovine blood, the markers did not change with shear stress stimulation except for plasma serotonin in heparin anticoagulated blood. These findings suggest that bovine blood is more resistant to shear-mediated platelet activation. This difference should be taken into account because understanding and correlating the interspecies differences of platelet reactivity may be crucial in optimizing the design of MCS devices both *in vitro* and *in vivo*.

In the literature, it was shown that more recent studies combined predictive and experimental approaches. Jesty et al., (1999) developed an acetylated prothrombin-based assay where the platelet activation state (PAS) was calculated on the rate of thrombin generation (formerly known as PF3 activity). Bluestein and colleagues preformed several studies using *in vitro* VAD loops or a modified cone-plate viscometer in an attempt to address the lack of information regarding the correlation between shear stress profiles and correspondent level of platelet activation (Jesty et al., 2003; Nobili et al., 2008; Sheriff J, Bluestein D et al., 2010). Human platelets were circulated in flow loops containing a variable length of 0.97 mm PTFE tubing (Small Parts Inc, Miami Lakes, FL) to mimic stenotic flow conditions. The exposure to

shear stress within the loops was adjusted by independently varying flow rate, viscosity, and time of exposure to shear. The results showed that the stenotic conditions dramatically increased the level of platelet activation compared with the non-stenotic control. The level of platelet sensitivity compared with different shear stress conditions was instead investigated using a cone-plate viscometer. In the study of Nobili et al. (2008), platelets were uniformly exposed to flow shear representing the lower end of the stress levels encountered in devices, and platelet activity state (PAS) was measured in response to six dynamic triangular and square shear stress waveforms representing repeated ideal passages through a device. PAS results was then correlated to predictions extrapolated from a damage accumulation model. Experimental results demonstrated an increase in PAS when the "relaxation" time between pulses was decreased, showing a good agreement with the model predictions. Finally, most recent studies performed by Sheriff and co-workers have investigated the behavior of platelets after higher level of shear stress exposure (Sheriff J, Bluestein D et al., 2010; Sheriff et al., 2013). In their protocols, purified platelets were exposed to short periods (5 – 40 s) of high-shear stress (up to 70 dyne/cm^2), and then exposed to longer periods (15 – 60 min) of low shear. Their activation state was measured using the PAS assay. Platelets shortly exposed to an initial high shear stress (e.g., 60 dyne/cm^2 for 40 s) showed a little activation, but when exposed to subsequent low shear stress, they activated at least 20-fold faster than platelets not initially exposed to high shear. The results showed thus that platelets exposed *in vitro* to shear beyond a defined threshold are primed for subsequent activation under normal cardiovascular circulation conditions, and they do not recover from the initial high shear insult. These results are of vital importance to understand the mechanisms behind VAD-mediated platelet activation. In fact, the possibility to correlate shear exposure and platelet activation have allowed to create powerful experimental predictive models with which may be characterized not only different devices but also new antiplatelet agents.

2.4.4 Bench devices for studying platelet response to shear stress

Implantable blood recirculating devices, artificial hearts and heart valves are currently used as life-saving alternatives for people affected by severe cardiovascular diseases. However, such devices require complex anticoagulation therapies that in turn can provoke post-implant complications, among which hemorrhage is the most prominent. Moreover, it is worth

mentioning that the risk for cardio-embolic stroke is not completely canceled by the anticoagulation therapy.

More than one factor contribute to the efficacy of cardiac recirculation devices: their biocompatibility is extremely important, but also an optimized geometric design is a necessary requisite to avoid flow-induced trauma to blood. Research works over the years demonstrated a definite link between fluid shear stress-induced damage and activation of blood constituents such as platelets and red blood cells. Recently, irregular flow patterns arising around complex geometries (some examples are the hinge regions of mechanical heart valves [MHV] or VAD internal geometries) have been characterized by numerical calculations and non-invasive flow measurements, which proved their contribution in causing flow-induced thromboembolism (TE). Since the results of such numerical analysis should be verified by experimental data, it is necessary to develop *in vitro* systems able to replicate specific flow conditions, including the ones causing the maximum thrombogenic damage. For this purpose, different devices have been built to perform bench studies on shear-mediated platelet activation. Among the methods used in literature, optical Light Transmission Aggregometry (LTA) is often applied (Born G, 1962). It consists in a method able to measure platelet activation in association with different chemical agonists. This assay requires a citrated blood sample, which after centrifugation results in platelet-rich plasma (PRP, with a platelet count of 200–300 x 10^9/L). To perform the assay, the platelet-rich plasma is positioned in an aggregometer cuvette along with a platelet agonist, such as ADP, arachidonic acid or collagen, to induce platelet aggregation. As the platelets aggregate, the turbidity of the PRP in the cuvette decreases. Thus, LTA allows to quantify this phenomenon by measuring the increased light transmittance over time.

During the last decades, not only methods able to measure platelet activation and aggregation, but also devices capable of subjecting platelets to shear stress and measuring their reactivity have been extensively described (Hellums JD, 1994). Among them, cone-plate viscometers (CPV) have been used in many studies to apply to a fluid sample a uniform and well-characterized shear field. The indicators of platelet activity are usually measured after an exposure time that can range from many seconds to a few minutes. Since the early 1980s, this type of viscometer has been extensively used to quantitatively measure platelet activation, blood viscosity and RBC damage (e.g. hemolysis) (Giorgio et al., 1988). The CPV consists of a rotating cone that is located on top of a flat base-plate, with the fluid of interest interjected between the conical surface and the base-plate. The angular separation between the former and the latter is typically 0.5°–3°. This allows to guarantee a uniform shear stress on the fluid

of interest, independent from both the radial position and the gap clearance. In cutting-edge versions of the CPV, the rotation of the cone can be dynamically controlled with a motor connected to a programmable interface (Girdhar et al., 2008). Additional advantages of the cone-and-plate viscometer with respect to traditional ones are (1) the ability to place a monolayer of cells on the plate to examine the platelet adhesion behavior and (2) the presence of an open suspension for platelets, which allows for rapid removal of the analyzed liquid. A CPV device endowed with a 0.5° rotating cone for shearing platelets was used by Shankaran and colleagues (Shankaran et al., 2001) (model VT550; Haake, Paramus, NJ). The main objective of such work was to examine platelets association properties with von Willebrand factors in suspension. With this device, the authors claimed that the minimum shear stress requirement for platelet activation, either in case of isolated platelets or in case of whole blood, is approximately 80 dyne/cm^2. The most recent studies use computer programmed viscometers in order to replicate *in vitro* the fluid dynamic conditions that blood encounters while passing through cardiac assist devices (Sheriff J, Bluestein D et al., 2010; Sheriff et al., 2013). The possibility of replicating high shear stress levels inside these viscometers represents a break-through in bench studies, but it involves the risk of formation of secondary flows that may affect the experimental conditions (Sutera et al., 1988). Bluestein's group in Stony Brook (New York) have described a computer controlled cone-plate viscometer able to replicated the flow shear stress extracted from commercial VADs. This device, called HSD, was designed to ensure the shear stress to be uniform through the device, with a modified Reynolds number kept below the limit of secondary flow effect ($Re < 0.5$) (Sdougos et al., 1984). The HSD, used for the studies of the present dissertation, carries significant improvements with respect to the most commonly used CPV and will be extensively described in the following paragraph.

2.4.5 The Hemodynamic Shearing Device

As described in Paragraph 2.3, DTE method allows to subject platelets to realistic shear stress profiles obtained from complex computational fluid dynamic (CFD) analysis performed within different cardiac devices (Figure 2.4). This technology facilitates optimization of devices performing first *in silico* analysis in the modeling domain (in which virtual design modifications are examined), followed by experimental emulation of the device specific stress loading histories (waveforms) *in vitro* using the hemodynamic shearing device (HSD). The

subsequent platelet activation after shear exposure is monitored using the PAS assay, already described in literature (Jesty et al., 1999), that will be discussed in chapter 3.

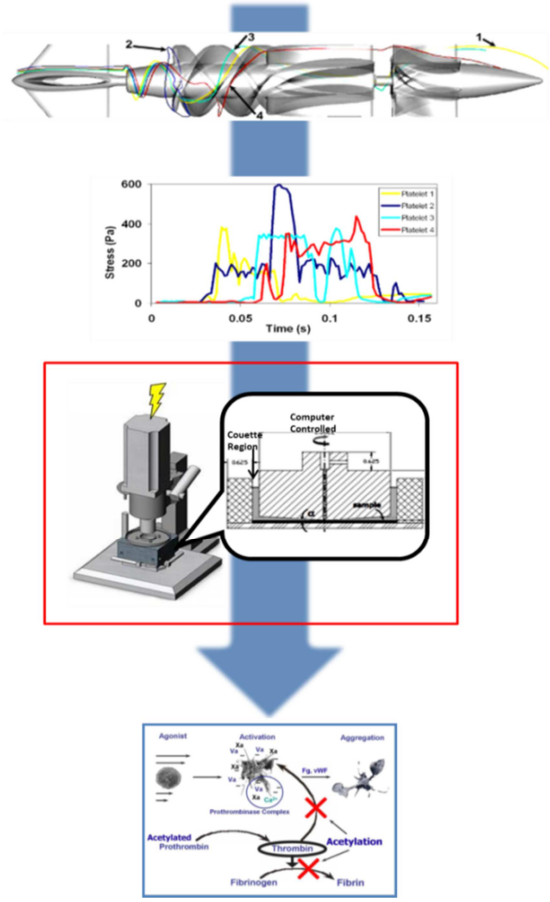

Figure 2.4. DTE Methodology. From *in silico* modelling to extraction of shear stress profiles tested with HSD and analysis of platelets activation via PAS assay. Adapted from (Girdhar et al., 2012).

The HSD is an innovative tool for measuring the dynamic response of cells under fluid shear conditions and represents a significant improvement over the traditional and more commonly used cone-plate viscometers (CPV). Indeed, most CPV systems generate only constant shear-stress over time. However, platelets flowing through devices are exposed to dynamically varying shear levels. The main innovative and peculiar characteristic of HSD is its capability of reproducing shear stress profiles derived from CFD analysis able to extract trajectories of individual platelets through cardiac assist devices. Dynamic shear stress waveforms can be programmed in BASIC by using the supplied Motion Architect software, before being transferred via a ZETA6104 micro-stepping drive to the HSD (Sheriff J, 2010). The HSD realistically emulates the effects of a cardiac device, since it is able to perform particular flow conditions. In the HSD, platelets are uniformly exposed to various dynamic loading waveforms and their resultant activity is measured. More details on the procedures to extract

the shear stress profiles from CFD simulations within VADs and to reproduce them within the HSD are reported in chapter 3.

Studies involving the analysis of platelet activation using the PAS assay after exposure to shear within the HSD can be used to complete *in vitro* bench investigation performed using actual VADs. In fact, during studies where PAS assay is coupled with VAD systems, measurements are performed of bulk properties of platelets sampled from circulation loops, without any *a priori* knowledge about the stress histories that these platelets were subjected to during the passages in the device (besides their circulation time when they are sampled). On the other hand, in the HSD the entire platelet population is uniformly exposed to prescribed dynamic shear stress waveforms. This is performed by rapidly changing the cone rotational speed. These two systems complement each other: while the VAD can provide a measure of the platelet activity under flow conditions in devices, the HSD brings out the effects of platelet activation in the so called "hotspots", using the stress loading histories along the trajectories defined by CFD.

The HSD consists of a programmable high-torque servo motor-controller system (Baldor Electric Company, AR) that drives the cone in the cone-Couette viscometer setup, as shown in Figure 2.5.

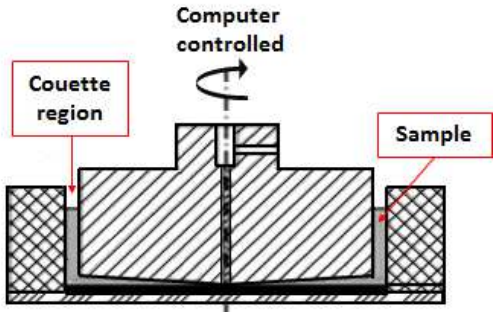

Figure 2.5. A schematic view of the HSD, a computer-controlled cone-plate and Couette viscometer that is capable of emulation dynamic shear stress conditions. Adapted from (Girdhar et al., 2012).

The HSD was specifically designed to guarantee a uniform flow field within the device. According to this feature, shear stresses are also uniform, thereby equating the shear stresses in both cone-plate and Couette regions (Nobili et al., 2008; Girdhar et al., 2008), as described in the following equation:

$$\tau_{Cone\text{-}Plate} = \tau_{Couette} \rightarrow$$

$$\mu\frac{\omega}{\alpha} = 2\mu\frac{R_o^2 R_i^2}{R_o^2 - R_i^2}\left(\frac{1}{r^2}\right)\Bigg|_{r = R_i} \Rightarrow \alpha = \frac{1}{2}\left[1 - \left(\frac{R_i}{R_o}\right)^2\right]$$

(small α, $(R_i/R_o) \rightarrow 1$)

This characteristic of the HSD removes possible “dilution” effects and variable shear stresses, which are instead present in capillary flow loops. The parameters of the equation are the following: μ, indicating the viscosity, has a value of about 1 cP, as measured for gel-filtered platelets (GFP) in a modified Tyrode’s buffer at 37°C; ω, the angular velocity of the cone, varies depending on the shear rate of interest; the cone angle α is equal to 1°; finally, R_o is the inner radius of the ring and R_i the outer radius of the cone. The cone angle determines the shear rate in the upper annular region (Sheriff J, 2010).

It is worth mentioning that the uniformity of the shear stress could be affected by periodical acceleration and deceleration exerted by the device. Nonetheless, this risk can be prevented by ensuring specific conditions. In particular, the modified Reynolds number $\tilde{R} = (r^2\omega\alpha^2)/12v$, has to be below 0.5, which is the limit of secondary flow effects, condition respected in the HSD (Sdougos et al., 1984, Shankaran et al., 2001).

This device gives the user the possibility to change the shear stress with step-like patterns (3 ms resolution). The accuracy in reproducing such shear conditions is extremely elevated (close to 100%). In fact, the driving motor includes an encoder that allows to record the actual velocity profiles reproduced by the device. Those profiles can be used as a feedback control and compared to the CFD simulations to check whether discrepancies between input and output velocity waveforms are present. The experimental parameters can thus be adjusted to reproduce the expected stimulation with higher precision (Xenos et al., 2010).

The constructive materials of the platelet-contacting surfaces of the HSD consist in ultra-high molecular weight polyethylene (UHMWPE) treated with silicone (in heptane solution, Sigmacote, Sigma-Aldrich, St. Louis, MO) in an attempt to eliminate the effect of platelet-surface interactions on platelet activation. The HSD can be heated at 37°C in order to replicate temperature inside the body. The volume of platelets used during the experimental campaign has to be high enough so sufficient volume remained when multiple platelet samples are taken, but not too much, thus ensuring that no spilling occurs at high cone velocities (Sheriff J, 2010). At the defined time points, the platelets are withdrawn from the Couette region using pipette tips, before being processed for the PAS assay.

2.4.6 The syringe-capillary pump system for the study of platelet activation under hyper shear conditions

Despite many advantages, the current version of the HSD allows a sustained maximum shear stress of 70 dyne/cm^2, far from the actual shear stresses experienced by platelets within VADs, which can reach up to thousands of dyne/cm^2 (Girdhar et al., 2012). In an attempt to overcome this limitation, a different device, called syringe-capillary shearing device (SCSD), can be used to reach hyper shear stress during the experiments. This device, built in collaboration between Stony Brook University (Prof. D. Bluestein) and University of Arizona (Prof. M. J. Slepian) is a syringe-capillary pump system. Within the device, platelets are exposed to different shear stresses by cyclically passing through capillaries with defined geometries at a controlled flow rate (Figure 2.6). The main advantage of using this device compared with HSD is the ability to reach hyper shear stress conditions (up to 1400 dyne/cm^2) with little adjustment of flow rate and geometrical modification of the capillaries coupled with the pumping system. In fact, to increase the shear stress peak obtainable within the HSD, an additional expensive and complex control system should be added to the device. On the other hand, this solution entails the risk of reducing the versatility of the already described rotational viscometer.

Despite the advantages of the SCSD, no controlled dynamic shear patterns extracted from CFD simulations can be reproduce using this device. For this reason, combined studies with the HSD has to be performed to investigate the behavior of platelets in a setting that is closer to the real conditions encountered *in vivo*. Furthermore, the use of capillaries for subjecting platelets to shear stress implies a non-uniformity in shear stress within the tubes.

The pump (PSD/8, Hamilton, Reno, NV) used in the SCSD is composed of 2 stepper motors: the first drives the 5 mL glass syringe with a Luer fitting (Hamilton, Reno, NV) via a connected plunger, while the second controls the opening and closing of the input/output valves (Figure 2.6). All the surfaces that contain blood are composed of PVC or PTFE, thereby reducing platelets activation or interaction with them. Only the syringe is made of glass. To prevent the platelets from sticking to the surface, the glass syringe is coated with Sigmacote (Sigma- Aldrich, St. Louis, MO) daily. The platelets have to be exposed just for very brief durations to the ceramic valve ports, in a way that they have minimal activation effects on them (Sheriff J, 2010). The maximum volume of sample that can be used for each experiment is 5 ml.

The diameter of the tubes used for the experiments range from 1.6 mm, which corresponds to the lowest shear stress condition, to 0.4 mm for the highest (1440 dyne/cm^2).

In one cycle within the device platelets are exposed to the actual shear stress for variable time intervals between 0.044 to 0.212 s. To allow platelets to experience shear stress for prolonged period of time, platelets are recirculated within the device, from a minimum of 12 to a maximum of 477 loops, according to the experiment. The device uses an inflow tube to refill the syringe pump after shear exposure during each cycle (Figure 2.6). The inflow consists in a silicon tube with inner diameter ϕ = 2 mm and length L = 200 mm. Flow rate within the inflow tube and the geometrical characteristics of the conduit allow to consider the shear stress in that section almost irrelevant compared to shear stresses experienced by platelets passing through the outflow capillaries (140 – 1440 dyne/cm^2). Further detail on the parameters used with SCSD are reported in chapter 4.

Downstream of the outflow valve port (Port 5) there is a pressure transducer (range: 0 – 100 psi, Omega Engineering Inc., Stamford, CT), which has to ensure that the pressure drop across the length of the test tubing does not exceed the limit for the valve ports of 100 psi. LabView 9 (National Instruments, Austin, TX) is used to control the commands of the system. Furthermore, a USB data acquisition (DAQ) device (National Instruments, Austin, TX) allows to download the pressure measurements into the control software.

A deep description of the fluid dynamics equations that characterize the operating principles of the SCSD can be found in (Sheriff J, 2010).

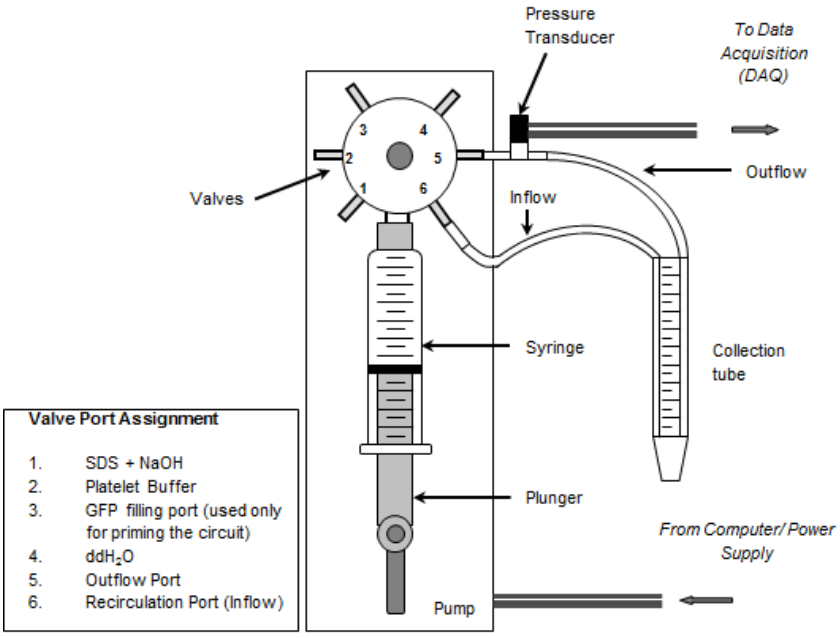

Figure 2.6. The syringe-capillary shearing device, a linear flow system for reproducing hyper-shear conditions. Adapted from (Sheriff J, 2010).

2.5 Antiplatelet Therapies

2.5.1 Mechanisms of action of antiplatelet therapies

Because of the central role of platelets in cardiovascular atherothrombosis, different antiplatelet drugs have been developed in an attempt to block most of the mechanisms behind platelet activation, thus allowing physicians to be able to create personalized antithrombotic therapies (Jackson SP, 2011). Antiplatelet therapy mainly includes ADP $P2Y_{12}$ receptors antagonists (thienopyridines as ticlopidine, clopidogrel, ticagrelor), cyclooxygenase [COX-1] inhibitors (aspirin), GPIIb-IIIa antagonists (eptifibatide) or phosphodiesterase receptor inhibitors (dipyridamole, pentoxifylline, cilostazol)(Michelson AD, 2011)(Figure 2.7).

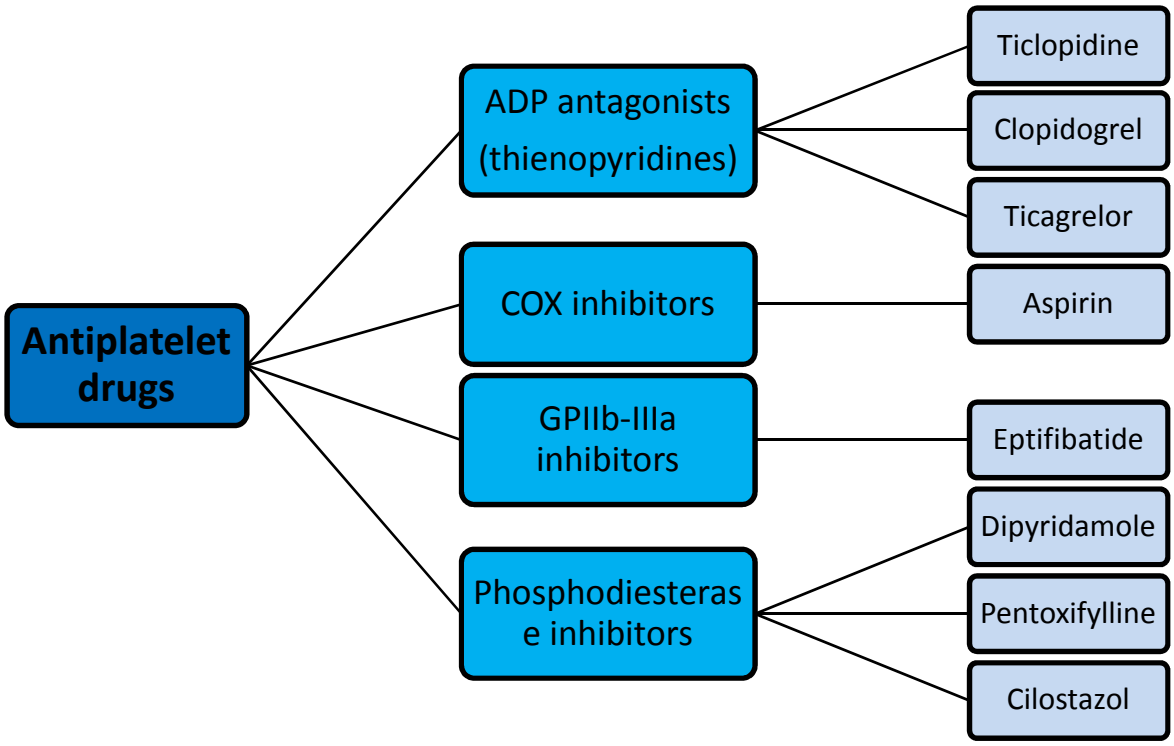

Figure 2.7. Classification of the main antiplatelet agents.

The mechanisms with which these different drugs act as antiplatelet agent are very different, as is described in Figure 2.8 (Kalantzi et al., 2012).

Both COX inhibitors and thienopyridines selectively inhibit a single pathway of platelet activation: aspirin affects the thromboxane A_2 (TxA_2) pathway by irreversibly inhibiting COX-1, while thienopyridines affect the ADP pathway by antagonizing one of the two platelet ADP receptors, $P2Y_{12}$ (Cattaneo M, 2013). The good antithrombotic efficacy of

these drugs, despite their selective mechanism of action, is explained by the fact that both the TxA_2 pathway and the ADP pathway contribute to the amplification of platelet activation and are essential for the full aggregation response of platelets. Phosphodiesterase inhibitors block one or more of the five subtypes of the enzyme phosphodiesterase (PDE), thereby preventing the inactivation of the intracellular second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by the respective PDE subtype(s) (Michelson AD, 2011). Finally, eptifibatide (trademarked as Integrilin) inhibit GPIIb-IIIa receptors, platelet membrane glycoproteins involved in binding fibrinogen, a key factor in the coagulation cascade (Clappers et al., 2007). Despite their effectiveness, clinical evidences report still a not negligible incidence of thrombosis in patients treated with currently available antiplatelet therapy (Jing et al., 2013). A possible explanation to this phenomenon is related to the intrinsic mechanisms of action of these drugs, developed to only chemically inhibit platelet activation. The non-physiologic fluid dynamic conditions that characterize blood flow within cardiac devices may actually overcome the protection effect provided by current antiplatelet agents.

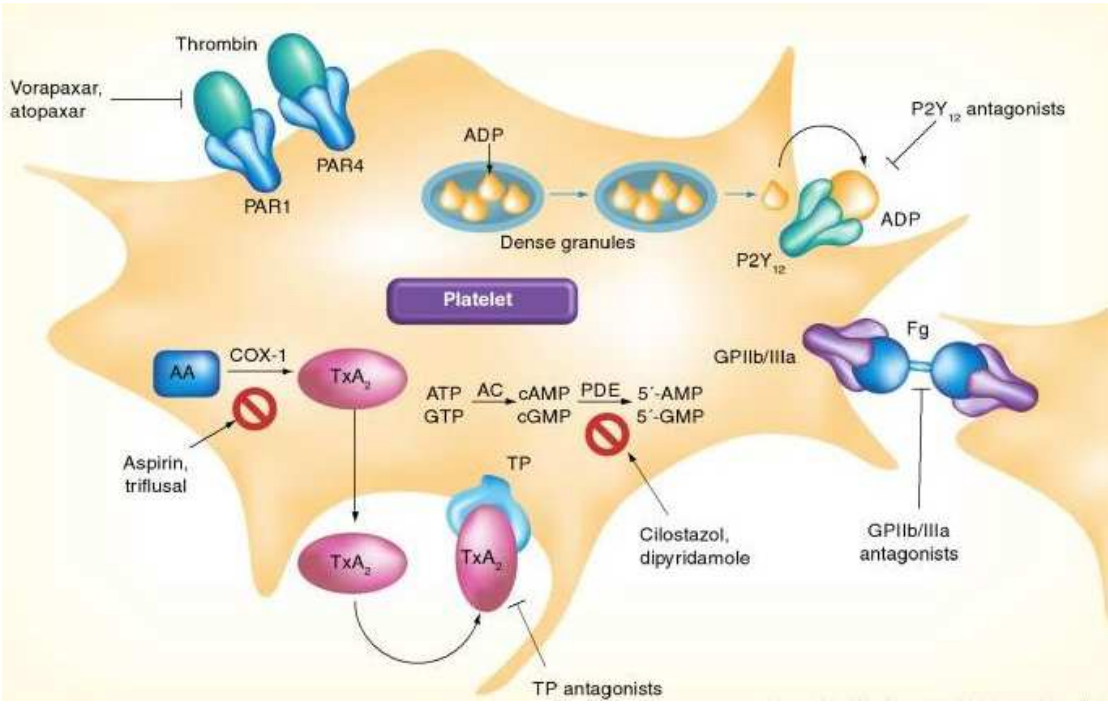

Figure 2.8. Sites of action of antiplatelet agents used in clinical practice. (Kalantzi et al., 2012)

2.5.2 COX Inhibitors: Aspirin

Aspirin (acetylsalicylic acid) produces its antiplatelet effects through the irreversible inhibition of the cyclooxygenase (COX) prostaglandin H-synthase-1 and H-synthase-2. Low-dose aspirin selectively inhibits COX-1, and high-dose aspirin inhibits both COX-1 and COX-2 (Patrono et al., 2008). Blocking the function of COX prostaglandins, aspirin inhibit the conversion of arachidonic acid to prostaglandin endoperoxide (EPOx, PG-G_2). This results in down-regulation of the synthesis of both thromboxane A_2 (responsible for platelet activation) and prostacyclin PGI_2 (responsible for endothelial deactivation) (Figure 2.9) (Gasparyan et al., 2008; Ensor et al., 2010; Pettigrew LC, 2001). Aspirin is eliminated by degradation to acetic and salicylic acids in the gut. The effective duration of action of aspirin is the lifespan of each platelet, approximately 8 to 10 days. Recovery of thromboxane A_2 generation is faster than the rate of platelet turnover after prolonged therapy; however, this action should not substantially affect the antiplatelet effects of aspirin. The common starting dosage of aspirin in the Multitargeted Antithrombotic Approach (MTA), a systematic thromboprophylaxis regimen, is 81 mg daily. In order to maintain suppressed platelet coagulability on LTA and normocoagulability on Thromboelastography (TEG) the dosage can reach the 325 mg/day (Ensor et al., 2010).

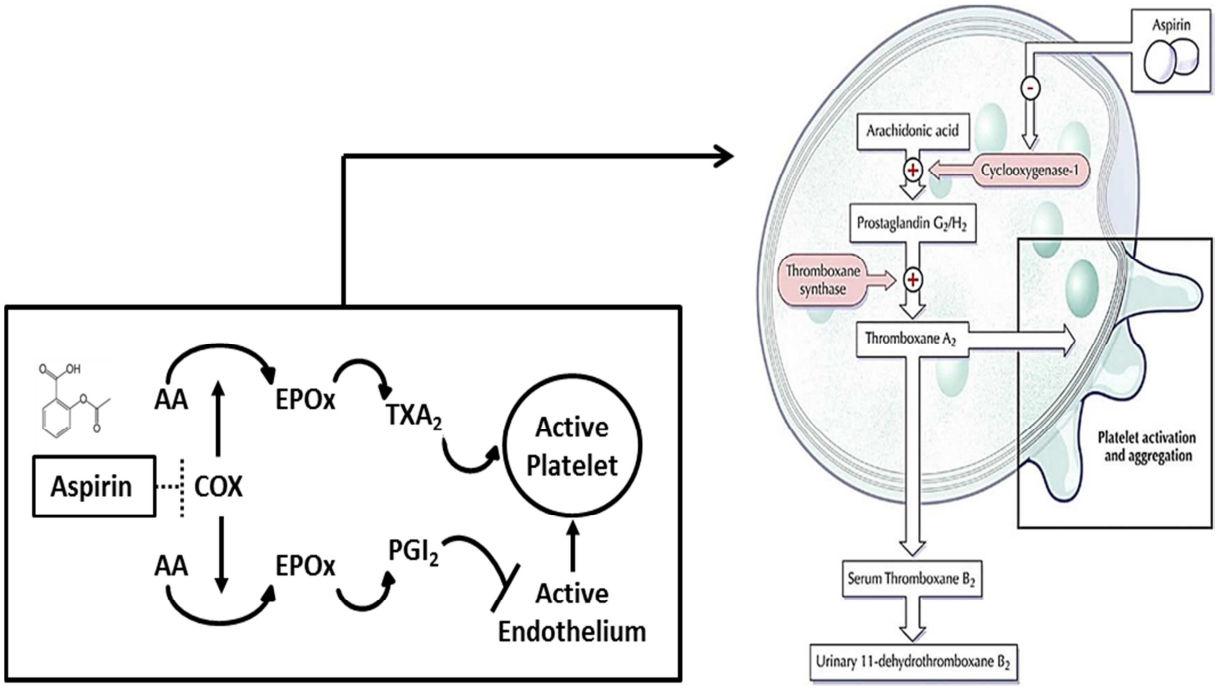

Figure 2.9. Mechanisms of action of Aspirin. Adapted from (Gasparyan et al., 2008; Ensor et al., 2010).

2.5.3 Phosphodiesterase inhibitors: Dipyridamole, Cilostazol and Pentoxifylline

Platelet activation and aggregation can be inhibited either by interaction with intracellular signaling pathways or by the blockade of membrane receptors (Gresele et al., 2011). Cyclic adenosine Phosphodiesterases (PDEs), by catalyzing the hydrolysis of 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP), two critical intracellular second messengers, limit the intracellular levels of cyclic nucleotides, thus regulating platelet function. In fact, cAMP and cGMP have a strong inhibitory activity on platelet functions; this is the reason why the action mechanisms provided by PDEs may be considered extremely important. Platelets possess three PDE isoforms (PDE2, PDE3 and PDE5), with different selectivity for cAMP and cGMP. Several nonselective or isoenzyme-selective PDE inhibitors have been developed, and some of them are now used in clinic as antiplatelet agents (Gresele et al., 2011). The phosphodiesterase inhibitors investigate in the present study are dipyridamole (trademarked as Persantine), cilostazol and pentoxifylline (Figure 2.10).

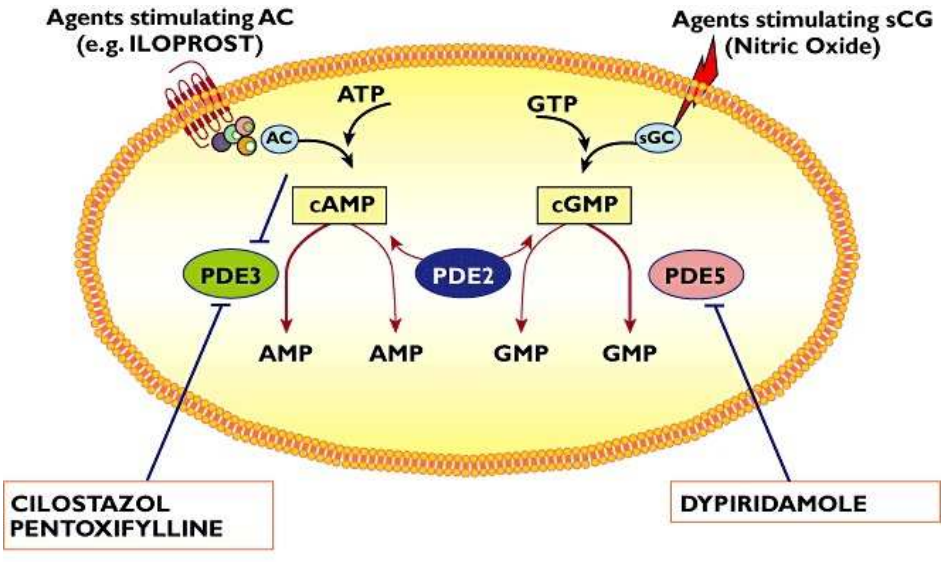

Figure 2.10. Schematic representation of a platelet and the mechanisms regulating intraplatelet levels of cyclic nucleotides (cAMP and cGMP). Mechanisms of action of pentoxifylline, cilostazol and dipyridamole (Gresele et al., 2011).

Dipyridamole (2,6-bis (diethanolamino)-4,8-dipiperidinopyrimido 5,4-d pyrimidine) affects platelet function by acting at several different targets. Primarily it inhibits the reuptake of adenosine by red blood cells. In this way it enhances the plasma concentration of adenosine, which is considered both a vasodilator and a platelet inhibitory nucleoside. Adenosine is released by tissues in the extracellular space as a breakdown product of ATP during ischemia, or by erythrocytes stressed by elevated shear forces. After its release, adenosine is recruited

by erythrocytes to keep plasma levels low. Inhibition of adenosine reuptake by dipyridamole is concentration dependent and reaches 90% at 1 mM of dipyridamole in whole blood, a concentration in the range of those attained after oral administration to humans (0.5–6 mM) (Klabunde RE, 1993).

Dipyridamole also acts as an inhibitor of PDE5 and PDE3, thus increasing intraplatelet cAMP and/or cGMP. The slight PDE3-inhibitory action of dipyridamole increases the effects of adenosine and PGI_2, both stimulators of adenylyl cyclase, leading to inhibition of platelet activation (Harker et al., 1983). By inhibiting PDE5, dipyridamole increase the production of vasodilator-stimulated phosphoprotein, an established marker of the NO/cGMP effects (Aktas et al., 2003), both *in vitro* and *in vivo*. Nitric oxide (NO) is a potent vasodilator and inhibitor of platelet activation. NO stimulates production of cGMP and activates cGMP-dependent protein kinase (G kinase), which leads to inhibition of Gαq-phospholipase C-inositol 1,4,5-triphosphate signaling and intracellular calcium mobilization for several important agonists, including thromboxane A2 (TXA2) (Wang et al., 1998). However, even if certified by several studies, the inhibition of PDE5 driven by dipyridamole is detectable *in vitro* only at concentrations of 100–200 mM, much higher than those attainable after oral administration.

Cilostazol, a 2-oxo-quinoline derivative (6-(4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy)-3,4-dihydro-2(1H)-quinolinone), inhibits PDE3 in platelets and smooth muscle cells. Its capacity of diminish intracellular calcium can cause smooth muscle cell relaxation and platelet activation inhibition (Gresele et al., 2011). One potential benefit of the use of cilostazol over conventional antiplatelet therapy is the short duration of its effect on platelet function. In fact, studies have demonstrated that platelets recover their normal reactivity within 12-16 hours after suspension of the drug (Iwamoto et al., 2003). Cilostazol inhibits the expression of monocyte chemoattractant protein-1, an initial trigger in the development of atherosclerosis, by increasing intracellular cAMP. Cilostazol reaches peak plasma concentrations (775 ng ml^{-1} = 2.09 mM) at about 2.4 h after oral administration. In plasma it is largely bound to proteins (95–98%), primarily albumin (Bramer et al., 1999). Specifically, metabolism of cilostazol occurs primarily via CYP3A5 and, to a lesser extent, CYP2C19, while < 1% of the administered dose is excreted unchanged in urine (Akiyama et al., 1985). After oral administration, cilostazol and its metabolites, 3,4-dehydrocilostazol and 4-trans-hydroxycilostazol, show a half-life of approximately 10 h, with a twofold accumulation during repeated administration (Schrör K, 2002).

Pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-xanthine) is a non-selective PDE inhibitor which reduce whole blood viscosity and improve erythrocyte deformability. Its

inhibitory effect was confirmed *in vitro* with platelet-rich plasma (PRP), even if tested at elevated concentration normally not used *in vivo*. (Nenci et al., 1981). However, because pentoxifylline platelet inhibitory effect was potentiated by PGI_2, it was suggested that its antiplatelet activity could be stronger *in vivo* compared to *in vitro* (Weithmann KU, 1980). Moreover, due to the contribution of adenosine uptake-inhibitory effect on erythrocytes, pentoxifylline was found more efficient in the inhibition of platelet aggregation in whole blood compared to PRP.

2.5.4 GPIIb-IIIa inhibitors: Eptifibatide

The human glycoprotein GPIIb-IIIa belongs to a family of cation-dependent adhesion molecules with a common heterodimeric structure known as integrins. The primary function of GPIIb-IIIa is to transmit bidirectional signals across the plasma membrane, assisting in this way platelet aggregation (Lippi et al., 2011). Platelet membrane activated receptor glycoprotein GPIIb-IIIa can bind fibrinogen, starting the final common pathway of platelet aggregation. Since the GPIIb-IIIa receptor is the most important integrins involved in platelet aggregation, the development of GPIIb-IIIa antagonists like eptifibatide (trademarked as Integrilin) has become an attractive strategy for antiplatelet therapy with an expected strong and specific effect (Figure 2.11). Eptifibatide is a cyclic heptapeptide inhibitor of GPIIb-IIIa. It has an active pharmacophore derived from the structure of barbourin, a GPIIb-IIIa inhibitor extracted from the venom of the southeastern pigmy rattlesnake. Like barbourin, eptifibatide is a specific and robust inhibitor of the GPIIb-IIIa receptor function. Its low affinity for other integrins makes eptifibatide a strong platelet aggregation inhibitor. Preclinical pharmacologic studies have established that eptifibatide can inhibit thrombosis effectively, reducing the risk of ischemic complications with only modest effects on bleeding time measurements (Phillips et al., 1997). Furthermore, large-scale clinical trials have demonstrated a clear clinical benefit and good safety profile in high-risk patients using Integrilin, especially those undergoing percutaneous coronary intervention (IMPACT II trial, 1997). However, adverse events related to thrombosis or bleeding are still reported in patients undergoing therapy with GPIIb-IIIa antagonists, suggesting how the drug reflects a variable inter-individual responsiveness.

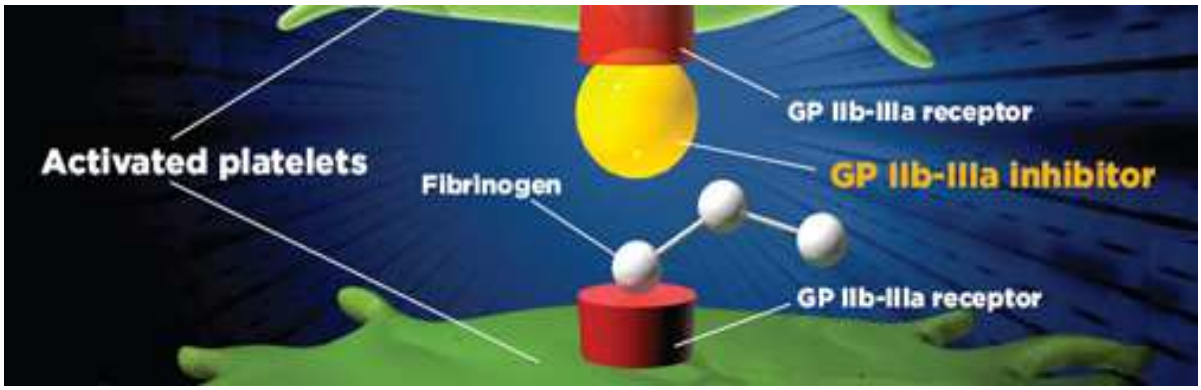

Figure 2.11. Mechanisms of action of Integrilin. Source: www.integrilin.com

2.5.5 ADP-receptor blockers: Ticagrelor

Thienopyridines, once metabolized both in the liver and intestines, can covalently bind to the $P2Y_{12}$ receptor causing irreversible platelet inhibition (Wallentin L, 2009).

Ticagrelor, often listed with thienopyridines inhibitors, reversibly interact with the platelet $P2Y_{12}$ ADP-receptor to prevent signal transduction and platelet activation (Figure 2.12).

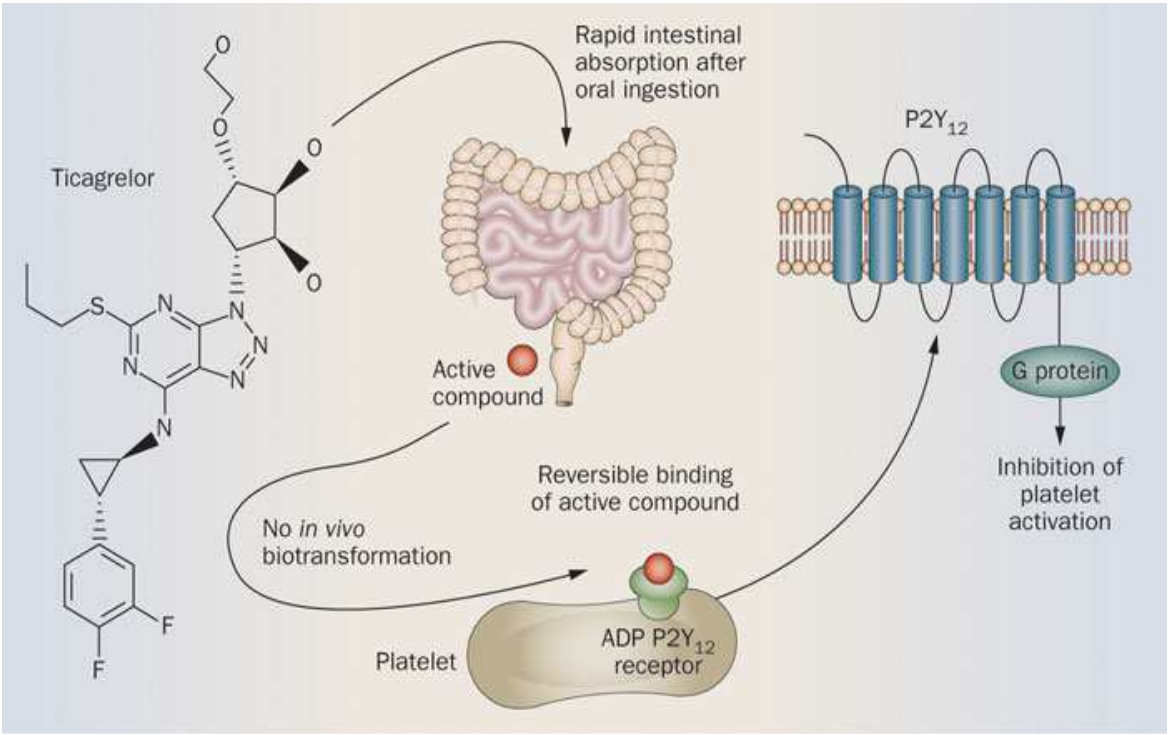

Figure 2.12. Mechanisms of action of ticagrelor. (Bhatt D.L., 2009)

$P2Y_{12}$ is considered the primary target of thienopyridines antiplatelet agents and plays an important role in regulating platelet aggregation and function. Inhibition of the platelet ADP $P2Y_{12}$ receptor has shown to be associated with a marked risk reduction of atherothrombotic events in high-risk settings, including patients with acute coronary syndromes and those undergoing mechanical circulatory support devices implant (Cattaneo M, 2011). Ticagrelor (AZD6140) is the first drug of a new chemical class called cyclo-pentyl-triazolo-pyrimidine, which is administered orally. (Van Giezen et al., 2009). Ticagrelor has shown a rapid, greater and consistent antiplatelet effect compared to other drugs both in preclinical and early-phase clinical studies. Furthermore, ticagrelor has been proved to be more effective in preventing ischemic events in acute coronary syndrome patients without an increased risk of protocol-defined major bleeding. Although characterized by a favorable safety profile, the use of ticagrelor has been associated with an increase in the rate of not procedure-related bleeding, compared with currently recommended treatment regimens (Capodanno et al., 2010). Absorption of ticagrelor occurs with a median t_{max} of 1.5 hours (range 1.0–4.0), with a nearly complete platelet inhibition (> 85%) at 2 to 4 hours following oral administration of 100 mg twice daily. Ticagrelor has a half-life of 7 to 8.5 hours, with the metabolite lasting up to 12 hours (Nawarskas et al., 2011).

2.6 Limitations of the *in vitro* experimental campaign on purified platelets

In the present study, the effect of different antiplatelet agents on shear-mediated platelet activation is investigated. In our experiments, platelets were exposed to shear stress using the HSD, the SCSD or VADs within flow loops. Platelet activity state was assessed using the PAS assay. The latter requires purified platelets as a working fluid and uses acetylated prothrombin to block the feedback action of the generated thrombin on the platelets, thus ensuring linear kinetics during the assay and quantitative measurement of PAS (Jesty J. et al., 1999).

Despite the novelty of the methodology adopted for the investigation, such approach presents some limitations. First, by using purified platelets instead of whole blood (WB), we neglected the contribution of red blood cells (RBCs), white cells (WCs) and plasmatic proteins to platelet activation process.

When flowing in WB, platelets transport is regulated by both diffusion and convection. RBCs have been shown to increase platelet diffusivity as a function of 1) shear rate and hematocrit through platelet margination and 2) shear rate through localized mixing and cell-cell collisions (Aarts et al., 1984; Goldsmith et al., 1986; Jordan et al., 2004). Margination refers to the lateral migration of platelets towards the wall in a conduit under laminar flow (Zhao et al., 2007). In laminar condition, blood viscosity and flow resistance decrease due to the RBCs axial migration (or Fahraeus effect) (Van Breughel et al., 1992). The highly deformable and biconcave-shaped erythrocytes migrate to the center of the flow and platelets are concentrated on the edge of the vessel wall (Du et al., 2014). Under high shear stress, erythrocytes slightly elongate in the direction of the flow; instead, when subjected to slow shear rates, erythrocytes tend to form aggregates and the peripheral distribution of platelets disappears (Du et al., 2014). Zhao and colleagues demonstrated that hematocrit plays a key role in lateral distribution of platelets under conditions of augmented shear stress. In fact, the authors observed no margination in the samples with zero hematocrit (Zhao et al., 2007). Furthermore, Du and colleagues stated that elevated hematocrit provides the transport of platelets and coagulation factors towards vessel wall, thereby increasing collisions of platelets with the vasculature and with one another. Since in our studies purified platelets were used in place of WB, according to what reported in the literature we implicitly assumed that platelet margination didn't play a major role during our experiments.

Andrews and colleagues pointed out that an increase in hematocrit results in increased blood viscosity (Andrews DA et al., 1999). Average WB viscosity is 3-4 cP. During our

experiments, we used purified platelets diluted in Hepes-modified Ca_2^{+}-free Tyrode's buffer, reaching a final viscosity of 1cP. Such reduced viscosity led to a lower level of shear stress within the devices used for our investigation. Therefore, when interpreting the results we should take into account that the shear stresses to which platelets were exposed were lower than in the WB environment.

RBCs can contribute to thrombosis and hemostasis by releasing substances that act as platelet agonists. In fact, when hemolysis occurs, RBCs mainly release hemoglobin, ADP and LDH in the blood plasma. These elements have different effect on thrombosis but they all enhance platelet activation (Helms CC et al., 2013).

WB includes also plasmatic proteins that contribute to platelet activation. In fact, some of the prothrombinase complex factors (factor Xa, Ca^{2+}), which are normally present in the blood plasma in their inactive form, play a key role in the platelet activation process by enhancing the conversion of prothrombin to thrombin. In our study, due to the absence of these factors in GFP solutions, this mechanism of activation was not considered. Nonetheless, the absence of the positive feedback on platelet activation provided by prothrombinase complex factors was necessary for the correct performance of PAS assay (more details can be found in paragraph 3.3.5).

The level of platelet reactivity may be influenced also by their concentration. In fact, prior studies examining platelet activation have shown that cross-talks between platelets can occur at high concentration (Schulz-Heik et al., 2005). Moreover, activated platelets can cause quiescent platelet activation (Sheriff J, Bluestein D et al., 2010). With the objective to maximize the sensitivity range of the PAS assay while limiting platelet collisions and cross-talks, platelet concentration used for our studies was maintained at a level of 20000 pl/µl (Jesty et al., 2003).

The limitations described above can be applied to the entire thesis except from chapter 5, where a few preliminary tests using WB as working fluid are reported.

It is worth mentioning that the goal of our work was not to study platelet behavior by replicating the biological environment present *in vivo*. Instead, we wanted to assess the effect of antiplatelet agents on platelet activation by measuring their ability to protect platelets under shear stress conditions. To this end, GFP was considered the best marker for the detection of platelet activation using the PAS assay.

As described in paragraph 2.4.5, during our experiments we used the HSD to subject platelets to different shear conditions. This device is able to reproduce shear stress waveforms

extracted from CFD simulations within VADs with a certain fidelity. Nonetheless, due to operating limitations, HSD is only able to reach shear peaks of 90 dyne/cm^2. This value is one order of magnitude lower compared to actual shear stresses within VADs (1-5 x10^3 dyne/cm^2). A factor scale of 15 was thus used as trade off to reduce the shear stress obtained from CFD simulations to levels feasible for the machine, but still interesting for the purpose of the study. Despite this limitation, the HSD represents a unique solution to mimic the extremely rapid dynamic behavior of the trajectories that platelets undergo while passing through VADs. The investigation of the effect of antiplatelet agents on platelet activation under such dynamic shear stress conditions is a pre-requisite for a full comprehension of the benefits and drawbacks of these treatments.

3

Do current antiplatelet agents truly protect platelets against actual shear exposure experienced in passage through ventricular assist devices?

This Chapter is based on:

1. Valerio L.*, Tran P.L., Sheriff J.*, Brengle W., Ghosh R., Chiu W.C., Redaelli A., Fiore G.B., Pappalardo F., Bluestein D., Slepian M.J. “Aspirin as a Means of Modulating Shear-Mediated Platelet Activation Over a Range of Shear Stress”. Journal of Thrombosis and Thrombolysis. *shared 1st author. Under submission.

2. Valerio L., Brengle W., Tran P.L., Hutchinson M., Chiu W.C., Sheriff J., Redaelli A., Fiore G.B., Pappalardo F., Bluestein D., Slepian M.J. “Do Current Anti-Platelet Agents Truly Provide Protection Against Shear-Mediated Platelet Activation in Mechanical Circulatory Support?”. JHLT. Under submission.

3.1 Introduction

In the present chapter the comparison of different antiplatelet agents is presented under both constant and dynamic shearing profiles, with the goal of understanding the behavior of these drugs in protecting shear-activated platelets.

Platelets provide the initial hemostatic plug at sites of vascular injury. They also participate in reactions that lead to atherosclerosis and pathologic thrombosis, especially when subjected to shear stress conditions as through MSC devices. Antagonists of platelet function, or antiplatelet drugs, have been used in an attempt to prevent thrombosis and to alter the natural history of atherosclerotic vascular diseases. The investigation of fluid dynamic conditions of blood passing through ventricular assist devices (VADs) results extremely important in order to verify the capacity of antiplatelet agents to prevent platelet activation. These pumps allow blood to circulate in the body when the heart has no longer the ability to accomplish its function. Despite their efficacy, VADs are burden with several complications as recurrent pump thrombosis, stroke or thromboembolic events. These events occur largely due to non-physiological flow past constricted geometries within the device, where platelets are exposed to rapid high shear stress spikes and exposure times. Under non-physiological conditions of flow through VADs, platelets are exposed to varying shear stress levels, turbulent stresses and prolonged residence times in pathological flow regions called “hotspots”, as well as repeated passages through the device that may precipitate activation, aggregation, and free emboli formation (Alemu et al., 2007). In an attempt to overcome these limitations, during the last decades both device manufacturers and clinicians have tried to develop more updated versions of VADs, as well as new pharmacological treatments. In particular, the latter have been considered of fundamental importance because of the actual impossibility to create devices that relieve patients from the need for complex pharmacological anticoagulation therapy. To date, current antiplatelet therapy, designed primarily to modulate biochemical pathways of platelet activation, have been poorly studied in combination with physical forces, such as shear profiles that mimic fluid dynamic condition inside VADs. The investigation of the effect of recent therapies in platelets protection from shear stress results fundamental to ensure a correct characterization of the products.

For this reason, the present study aims at investigate the effect of antiplatelet therapies on shear-induced platelet activation after exposure to both constant and dynamic shear stress profiles via the hemodynamic shearing device (HSD). The latter consists of a programmable

high-torque servo motor-controller system (Baldor Electric Company, AR) that drives the cone in the cone-Couette viscometer setup with the capability of expose platelets to highly-controlled dynamic shear stress patterns (Girdhar et al., 2008). Specifically, for what concerned the dynamic fashion, stress loading waveforms were extracted from detailed numerical flow modeling of the clinical used DeBakey VAD. Computational fluid dynamics (CFD) analysis allowed to model flow path in the VAD with a high fidelity two-phase FSI (fluid-structure interaction) simulation. These *in silico* analysis resolved all components of the stress tensor that are relevant to flow-induced thrombogenicity and subsequently traced down and captured the loading history of platelets in the flow field along trajectories that may drive them beyond the activation threshold.

Extreme cases of platelet stress loading waveforms can be tested in the HSD. For the purpose of the study, platelets were exposed *in vitro* to both established high ("hotspot") stress and low dynamic shear stress profiles and their degree of activation was characterized; then, we tested current anti-platelet agents, individually and in combination, to examine their ability to modulate platelet reactivity after being exposed to shear. These tests allowed us to assess the ability of common clinically-used antiplatelet drugs to inhibit platelet reactivity after shear exposure, so providing useful information to VAD's manufacturers, clinicians and pharmaceutical companies to determine the threshold over which physical forces overwhelm the drug's protective action.

3.2 Antiplatelet Agents: the effect of different mechanisms of action on platelet activation *in vitro* and *in vivo*

Traditionally, platelet function tests have been used for either screening or diagnosing platelet defects. Such tests are not only difficult to perform, but are also expensive, time consuming, and require relatively large volumes of fresh blood. Among them, several techniques are also employed to monitor the efficacy of pharmacotherapy in reducing risk of thrombosis (Harrison et al., 2007). The most common are Light Transmission Aggregometry (LTA), the Prothrombin Time (PT) test, the activated Partial Thromboplastin Time (aPTT) test, the International Normalized Ratio (INR) and flow cytometry (Wever-Pinzon et al., 2012; Slaughter et al., 2011; Sheriff et al., 2014). Furthermore, the growing importance of studying platelets in order to reduce the incidence of thrombosis has led to the development of point-of-care devices as Thromboelastography (TEG), platelet function analyzer (Mammen et al., 1998) and cone-and-plate(let) analyzer (Varon et al., 1998). However, a real standardized approach that objectively determines pharmacotherapeutic efficacy is still lacking (Von Ruden et al., 2012). Moreover, just few studies tried to investigate the effectiveness of antiplatelet therapies in response to shear stress, as the majority of drugs are intended as chemical-inhibitors rather than physical protectors (Sheriff et al., 2014). As mentioned in Chapter 2, antiplatelet drugs are divided into categories based on their mechanisms of action. In the present work, agents representing each of the principal classes were investigated under several shear stress conditions, with the objective of fully characterizing those different mechanisms. The latter, as well as the actual drugs studied in the present work are reported in Table 3.1.

Mechanisms of action	Antiplatelet Agents
COX Inhibitors	Acetylsalicylic Acid (ASA, **Aspirin**)
Phosphodiesterase receptor inhibitors	**Dipyridamole** (trademarked as Persantine) **Cilostazol** **Pentoxifylline**
GPIIb-IIIa receptor inhibitors	**Eptifibatide** (trademarked as Integrilin)
P2Y(12) receptor inhibitors	**Ticagrelor**

Table 3.1. Mechanisms of action of the antiplatelet agents investigated in the study.

Aspirin (ASA) inhibits platelet function by permanently acetylating the cyclooxygenase (COX) that forms prostaglandins. Blocking the function of COX prostaglandins, aspirin inhibit the conversion of arachidonic acid to prostaglandin endoperoxide (EPOx, PG-G_2). This results in down-regulation of the synthesis of both thromboxane A_2 (TxA_2 responsible for platelet activation) and prostacyclin PGI_2 (responsible for endothelial deactivation). Several studies have been conducted to study its effect alone or in combination with other antiplatelet agents both *in vitro* and *in vivo*. Burch et al. determined the sensitivity of platelets to aspirin in normal subjects by measuring [3Hacetyl] aspirin-susceptible cyclooxygenase in washed platelets obtained at various times after aspirin ingestion. The authors proved that a single 325-mg aspirin dose inactivated 89% of platelet cyclooxygenase. The inhibition persisted for 2 days, suggesting that oral aspirin also inactivated megakaryocyte cyclooxygenase. Thereafter, active enzyme returned with a time-course reflecting platelet turnover (life-span 8.2+2 days) (Burch J.W., 1978). The effect of aspirin has been always questioned, especially regarding shear-mediated platelet activation. In fact, Moake demonstrate that shear-induced platelet aggregation is mediated by ADP and large or extremely large vWF multimers and is poorly inhibited by aspirin. Authors described as the selectivity of its mechanism of action would indeed not protect platelets from high shear stress profiles like the ones experienced within VADs (Moake et al., 1988).

More recently an *in vitro* study conducted by Sheriff compared the efficacy of aspirin on platelets subjected to shear stress. In particular, the drug solution was either created in lab or was metabolized by human volunteers who were asked to ingest aspirin before blood donation (Sheriff et al., 2014). In the study presented, purified human platelets were circulated for 30 min in a flow loop containing the DeBakey VAD (MicroMed Cardiovascular, Houston, TX, USA). The results obtained proved that *in vitro* treatment with antiplatelet drugs such as aspirin is as effective as *in vivo* metabolized aspirin in testing the effect of reducing shear-induced platelet activation (Sheriff et al., 2014).

Another category of antiplatelet agents that have been investigated in the present study was the one of phosphodiesterase receptor inhibitors. Among those agents, great attention has been lately focused on **dipyridamole** (trademarked as Persantine), even if there is little clinical evidence that dipyridamole alone exerts an antithrombotic effect. Instead, several studies have been carried out in combination with aspirin. Dipyridamole associated with low-dose aspirin provides stroke risk reduction in patients with ischemic cerebrovascular disease (Gresele et al., 2011). This was confirmed in two large clinical studies: the first is ESPS2 (Diener et al., 1996; Lentz et al., 2000), in which 6602 patients with prior stroke or transient

ischemic attack were randomized to aspirin (25-50 mg daily), slow release dipyridamole (200 mg twice a day), the two agents in a combined formulation, or placebo; the second is the ESPRIT trial (Halkes et al., 2006), in which 2739 patients with previous transient ischemic attack were randomized to aspirin (30–325 mg day) with or without dipyridamole (200 mg twice daily). A meta-analysis supports the higher efficacy of combination therapy over aspirin alone (Verro et al., 2008). Based on these findings, the 2008 American College of Chest Physicians (ACCP) guidelines recommended dual therapy with extended-release dipyridamole plus aspirin over aspirin monotherapy for stroke prevention after a first transient ischemic attack or stroke (Adams et al., 2008).

In a further clinical study involving 294 patients 3 months after CABG, 192 patients were taking aspirin alone (75 mg/day) and 102 patients were taking both aspirin (75 mg/day) and dipyridamole (100 mg t.i.d.). No significant difference was observed between patients who were taking aspirin alone or in combination with dipyridamole in terms of hemostatic reaction of aggregation. Additionally, coagulation or clot stabilization did not differ between patients and controls. The effective dosage of aspirin for preventing thromboembolic events that emerged from the study was >300 mg/day (Ratnatunga C.P., 1992).

Among phosphodiesterase receptor inhibitors, **cilostazol** has been proved to inhibit both primary and secondary platelet aggregation induced by collagen, ADP, arachidonic acid and adrenaline (Kimura et al., 1985). It also suppresses the expression of P-selectin (Kariyazono et al., 2001, Inoue et al., 1999), TxB2 production, platelet factor 4 and platelet-derived growth factor release. Cilostazol inhibits shear stress-induced platelet activation *in vitro*, with an IC50 of 15.0 mM (Minami et al., 1997), and *ex vivo* is not dissimilar from ticlopidine plus aspirin. The effect of cilostazol was studied *in vivo* by Tsukikane. During this investigation, 211 patients with 273 lesions who underwent successful PTCA were randomly assigned to the cilostazol (200 mg/d) group or the aspirin (250 mg/d) control group (Tsuchikane et al., 1999). Cilostazol appeared to significantly reduce restenosis and target lesion revascularization rates after successful PTCA compared to aspirin, thus making this drug a powerful tool for platelet activation inhibition also in patients with VADs.

Pentoxifylline has also been studied by several research groups, either alone or in combination with other agents. Magnusson and colleagues found that this phosphodiesterase receptor inhibitor and its metabolites (rac-M1, R-M1, S-M1 and M4) significantly inhibit ADP induced platelet aggregation in whole blood *in vitro* (Magnusson et al., 2008). Pentoxifylline has also demonstrated a powerful effect *in vivo*, even if adjunctive treatment in combination with other antiplatelet agents (cilostazol and aspirin) is not associated with

increased platelet inhibitory effects in Diabetes Mellitus (DM) patients with coronary artery disease receiving dual antiplatelet therapy (DAPT) (Ueno et al., 2011).

The discovery that one of the final common steps in platelet aggregation is the binding of fibrinogen to the activated platelet integrin glycoprotein (GP) IIb/IIIa has opened the door to the development of novel and potentially more effective antithrombotic therapies like **eptifibatide** (trademarked as Integrilin). Nannizzi-Alaimo and colleagues showed that eptifibatide inhibited platelet aggregation by at least 80%. Integrilin also inhibited *in vitro* a soluble form of CD40 ligand (sCD40L), a prothrombotic and pro-inflammatory protein with GP IIb/IIIa binding activity and an established role in atherosclerotic lesion progression (Nannizzi-Alaimo et al., 2003). Moreover, in clinical studies as the phase III IMPACT-(Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis)-II trial and PURSUIT (Harrington RA, 1997) (Platelet GP IIb-IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy) trial, eptifibatide was found to reduce coronary events significantly in a broad range of low-, medium-, and high-risk patients with acute coronary syndromes, without significantly increasing the risk of bleeding or other complications (Scarborough RM, 1999).

$P2Y_{12}$ inhibitor **ticagrelor** has been studied *in vitro* to assess its efficacy. Ticagrelor is often listed with thienopyridines (ADP receptor inhibitors) and has similar indications for use but is not a thienopyridine. Tomizawa and colleagues showed that ticagrelor (3 and 10 mg/kg) has significant antiplatelet effects, even if its power diminished at 24 hours after the dosing. The proposed *in vitro* and *ex vivo* studies showed strong correlations between platelet aggregation and phosphorylation of vasodilator stimulated Phosphoprotein (VASP) for several $P2Y_{12}$ inhibitors comprising ticagrelor (Tomizawa et al., 2013), thus certifying the need to further investigate the ability of these agents to protect also shear-mediated platelet activation.

Despite these considerations, **several antiplatelet agents in combination with aspirin have been investigated.** In fact, studies have highlighted that the possibility of blocking more than one mechanism of platelet activation simultaneously would lead to a magnified protection effect.

Theoretically, inhibition of the two main amplification pathways of platelet aggregation, the ADP and the arachidonate/TxA_2 pathways, is superior to inhibition of either pathway alone in preventing thrombus formation (Cattaneo, 2004). Cattaneo has measured platelet function *in vivo* by monitoring the bleeding time, whereas, in most instances, platelet function *in vitro* has been measured by Light Transmission Aggregometry (LTA) or by other global techniques that evaluate primary hemostasis. All these techniques are sensitive to several variables.

Among these, platelet TxA_2 production, which is the pharmacological target of aspirin, is usually of marginal importance, and one should not expect aspirin to inhibit completely platelet functions that are not regulated by TxA_2 only. The author showed that measuring the bleeding time represents a highly inaccurate and poorly reproducible technique, which is dependent on several variables, including platelet function, platelet count, plasma factors, red blood cells, and the vessel wall. Cattaneo showed that aspirin does not prolong the bleeding time of many individuals because the inhibition of TxA_2-dependent platelet function can be easily outweighed by other variables that cannot be affected by aspirin. LTA measures the increase in light transmission through a platelet suspension that occurs when platelets are aggregated by an agonist. Arachidonic acid, being the precursor of TxA_2, is certainly a more suitable platelet agonist than ADP for studying the effects of aspirin (Taylor et al., 1992). However, the final platelet aggregation induced by arachidonic acid is the sum of the effects of synthesized TxA_2 and other agonists secreted by the platelet granules (Cattaneo M, 2004). Although the limitations of the tested methods, the author proved that aspirin and other ADP receptor inhibitors as ticlopidine and clopidogrel display good antithrombotic activity, especially if tested in combination with each other, despite several cases of patients' resistance to these agents that can affect their effectiveness.

The effect of COX or ADP receptors inhibitors has been also studied *in vivo*. Cooke and co-workers reported a study in which 60 patients were assigned randomly to ASA 325 mg/day, clopidogrel 75 mg/day, or both. After 10 days, platelet function was studied using LTA. The authors reported as clopidogrel provided stronger platelet inhibition than ASA with adenosine diphosphate as the agonist, and combination therapy resulted in greater inhibition than either inhibitor used alone ($p < 0.0001$). The use of ASA resulted in greater inhibition compared with clopidogrel with epinephrine ($p < 0.0001$) and collagen as agonists ($p < 0.0001$). The combination of ASA and clopidogrel appears superior to either agent alone in inhibiting platelet function (Cooke et al., 2006).

Furthermore, Alexander (1999) conducted a study that investigated the effect of aspirin on patients enrolled in the "Platelet IIb/IIIa in Unstable angina: Receptor Suppression Using Integrilin Therapy" (PURSUIT) trial (Alexander et al., 1999). In particular, he examined whether eptifibatide had a differential treatment effect in prior aspirin users. In a multivariable model, eptifibatide did not have a different treatment effect in prior aspirin users compared with nonusers ($p = 0.534$).

As emerged from this analysis, there are conflicting opinions regarding the effectiveness of antiplatelet therapies, especially for what concerns shear-mediated platelet activation. In fact, most of the current antiplatelet agents were originally developed as antipyretics (ASA) and vasodilators (cilostazol). Moreover, certain drugs like eptifibatide were developed as antiplatelet agents but never investigated in association with MCS devices. Thus, a complete investigation is necessary to provide a systematic study of this phenomenon and describe different scenarios that up to now have been only marginally discussed in literature. **The purpose of the present study was to investigate the effect of antiplatelet agents on shear-mediated platelet activation, with the objective of defining different properties of commonly clinical used drugs and finding a threshold over which their protecting effect may be overwhelmed by the physical forces that can be encountered within VADs.**

3.3 Materials and methods

3.3.1 Experimental concept

The experimental concept of this study is graphically described in Figure 3.1. After centrifugation of whole blood, from which platelet-rich plasma (PRP) was obtained, platelets were extracted using a gel chromatography column. Gel filtered platelets were subsequently diluted to a standard count of 20000 pl/µl using a modified Tyrode's buffer, then they were subjected to shear stress according to the experimental conditions. More details about the single procedures performed will be given in the following paragraphs.

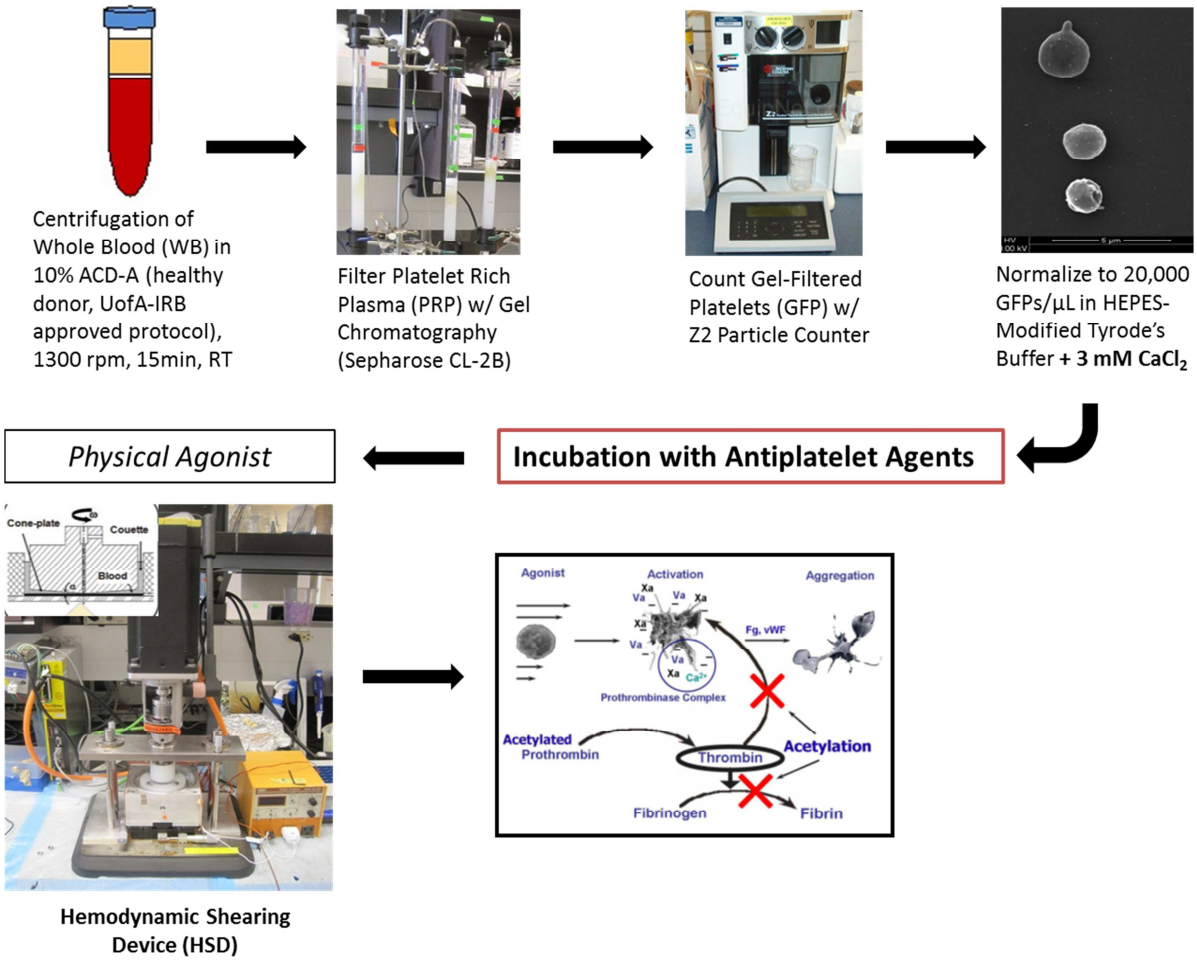

Figure 3.1. Overall Experimental Concept. Platelets were subjected to several experimental conditions in order to assess their reactivity to external stimuli. Details regarding the procedures performed are reported in Paragraphs 3.3.2 – 3.3.5.

3.3.2 Experimental protocol: platelet preparation

In the present chapter, the experimental protocol utilized to obtain purified platelet refers to a protocol described in previous studies reported in the literature (Sheriff J, Bluestein D et al., 2010; Schulz-Heik et al., 2005; Jesty et al., 1999). Nonetheless, our protocol has slight differences due to the instrumentation present in the laboratory facilities at University of Arizona, as well as depending on the experimental sets performed.

Briefly, informed consent was obtained from healthy adult volunteers of either sex as approved by the University of Arizona IRB. A volume of 30 ml of whole blood was drawn by antecubital venipuncture, collected in 3.8% trisodium citrate (ACD-A) (9:1,V/V) and centrifuged at 1300 rpm for 15 min.

The obtained PRP, which underwent a separation procedure from red blood cells, was gel-filtered through a 175-ml column of Sepharose 2B beads (60-200 μm diameter, 2% agarose; Sigma-Aldrich, St. Louis, MO) equilibrated in platelet buffer. The latter was a Hepes-modified Ca_2^{+}-free Tyrode's buffer, consisting of (final concentrations) 135 mM NaCl, 1 mM $MgCl_2$, 5 mM D(+)-glucose, 0.5 mM Na_2HPO_4, 1 mM $Na_3C_6H_5$ (Trisodium Citrate), 2.7 mM KCl, 0.1% bovine serum albumin (BSA), and 10 mM Hepes (Neuenschwander et al., 1988). NaOH was used to adjust the pH to 7.4. The result of the filtration process, i.e. gel-filtered platelets (GFP), was counted using Z2 Particle Counter (Beckman Coulter, Miami, FL) and diluted with platelet buffer to a concentration 20,000/μl, unless otherwise stated. After proper dilution, platelets were maintained flat and occasionally they were gently mixed while not used during experiments.

At this point of the procedure, the test solutions (4 ml) were incubated with or without the antiplatelet agents for 10 min at 37 °C before shear stress exposure via HSD.

3.3.3 Drugs preparation for the experimental campaign

As mentioned in Paragraph 3.2, the antiplatelet agents investigated in the present study were aspirin, dipyridamole, cilostazol, pentoxifylline, eptifibatide and ticagrelor.

Following a protocol already described in the literature (Sheriff et al., 2014), aspirin was dissolved in a sodium bicarbonate solution composed of 180 mg ASA (Sigma-Aldrich Corp.), 270 mg of citric acid and 349 mg of sodium hydrogen carbonate in 10 ml of double-distilled water (ddH_2O), thus obtaining a final concentration of 0.1 M.

Dipyridamole was obtained from a stock of Persantine® containing dipyridamole (5 mg/ml), polyethylene glycol (PEG) 6000 (50 mg/ml) and tartaric acid (2 mg/ml) dissolved in water. This injection solution was then diluted to obtain the concentrations desired.

A solution containing cilostazol was obtained after pulverization of commercial tablets of cilostazol (50 mg, Apotex Corp.). The powder was then dissolved into 10 ml of dimethyilformamide (DMF), obtaining a final concentration of cilostazol in the stock solution of 0.1 M.

280 mg of pentoxifylline (Sigma-Aldrich Corp.) were dissolved in 10 ml of ddH_2O for a final concentration of 0.1 M. The pH of the stock solution was rebalanced to 7.4 using a solution of NaOH 0.1 M.

The eptifibatide (Integrilin®, Merck & Co.) infusion vial used for the experiments was supplied as a 100 ml vial containing 0.75 mg/ml of eptifibatide.

Finally, ticagrelor was obtained after pulverization of commercial tablets of Brilinta® (90 mg, AstraZeneca). The powder obtained was dissolved in 10 ml of ddH_2O obtaining a final concentration of ticagrelor of 0.1 M.

Actual drug concentrations used during the experimental campaign are reported in Table 3.2.

Antiplatelet Agent	Working Dosage	Stock Concentration	Working Volume - 4 ml
Acetylsalicylic Acid (Aspirin)	25 µM (~ 81 mg/day)	0.1 M	1 µl
	125 µM (~ 325 mg/day)		5 µl
Eptifibatide	0.25 µg/ml	0.75 mg/ml	1.33 µl
Pentoxifylline	100 µM	0.1 M	4 µl
Dipyridamole (Persantine)	5 µM	5 mg/ml	2 µl
	10 µM		4 µl
	25 µM		10 µl
Cilostazol	50 µM	0.1 M	2 µl
Ticagrelor	10 µM	0.1 M	0.4 µl
	100 µM		4 µl

Table 3.2. Concentration of Antiplatelet agents used in the study. References: aspirin: Ensor et al., 2010; Burch et al., 1978; eptifibatide: correct dosage calculated from cath lab experience; pentoxifylline: Kullmann et al., 1993; dipyridamole: Meester et al., 1998; cilostazol: Kariyazono et al., 2001; ticagrelor: Tomizawa et al., 2013.

With the exception of eptifibatide and dipyridamole, the other agents tested in the present study were diluted before the experiments following specific protocols. In order to test the solution made during the preparation of the drugs and to control the effectiveness of the drug used in the *in vitro* campaign, an experiment involving aspirin was conducted. As described in Paragraph 3.2, aspirin inhibits the conversion of arachidonic acid (AA) to prostaglandin endoperoxide (EPOx, PG-G_2), thus blocking the function of COX prostaglandins. We therefore tested the reactivity of GFP pre-incubated with ASA after spiking with a certain concentration of AA. In particular, after incubation of platelets with or without aspirin (125 µM) for 10 min, AA (25 µM) was introduced in the sample tubes and PAS assay performed after 0, 10 and 30 min. This type of experimental verification was due on aspirin because it was easily possible to correlate the effect of the drug, whose mechanism of action directly inhibits the AA action, with the platelet activity state derived from the PAS assay. The results obtained are reported in Figure 3.2 and Table 3.3.

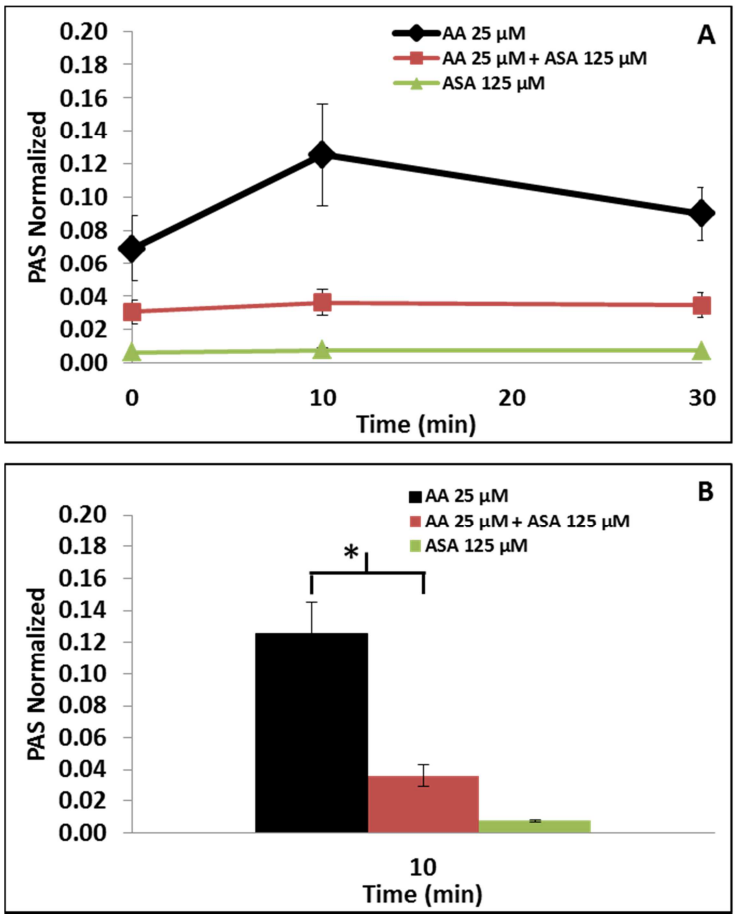

Figure 3.2. The effect of ASA on platelets spiked with AA. A. Platelets activation in time after treatment with AA alone (Black), ASA and then AA (Red) or ASA alone (Green). B. Platelets activation level after 10 min in the same conditions as A. * $p < 0.01$.

Experimental Conditions	PAR 0-10 min [s^{-1}]
AA alone - 25 µM	0.000095
AA + ASA - 25 µM + 125 µM	9.17E-06
ASA alone - 125 µM	1.83E-07

Table 3.3. Platelet Activity Rate (PAR) calculated for each experimental conditions between 0 and 10 min.
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As visible in Figure 3.2, the sample treated with AA showed an increase in platelet activity state between 0 and 10 min, compared to samples pre-treated with ASA. Both PAS values (0.12 vs 0.04, $p < 0.01$) and PAR calculated between 0 – 10 min (Table 3.3) attested that the protective effect of the drug was maintained after 10 min of incubation with AA. PAS values of the AA-treated platelets showed a slight non-significant decrease after 10 min of incubation. This trend was considered not important for the assessment of aspirin effectiveness and suggest that after 10 min of exposure to AA, platelet samples were no longer suitable for our investigation. This test confirms the suitability of PAS assay for testing the effect of chemical mediators and inhibitors. Moreover, the results suggested that 10 min of pre-incubation with the antiplatelet agent was an appropriate amount of time for drug-platelet interaction prior to shear stress exposure.

3.3.4 Shear stress profiles: constant and dynamic waveforms isolated from DeBakey VAD

As described in chapter 2, the DTE method allows to subject platelets to realistic shear stress profiles obtained from complex computational fluid dynamics (CFD) analysis performed within different cardiac devices (Girdhar et al., 2012). This technology facilitates optimization of devices performing first *in silico* analysis in the modeling domain (in which virtual design modifications are examined), followed by experimental emulation of the device specific stress loading histories (waveforms) *in vitro* using the HSD (Figure 3.3).

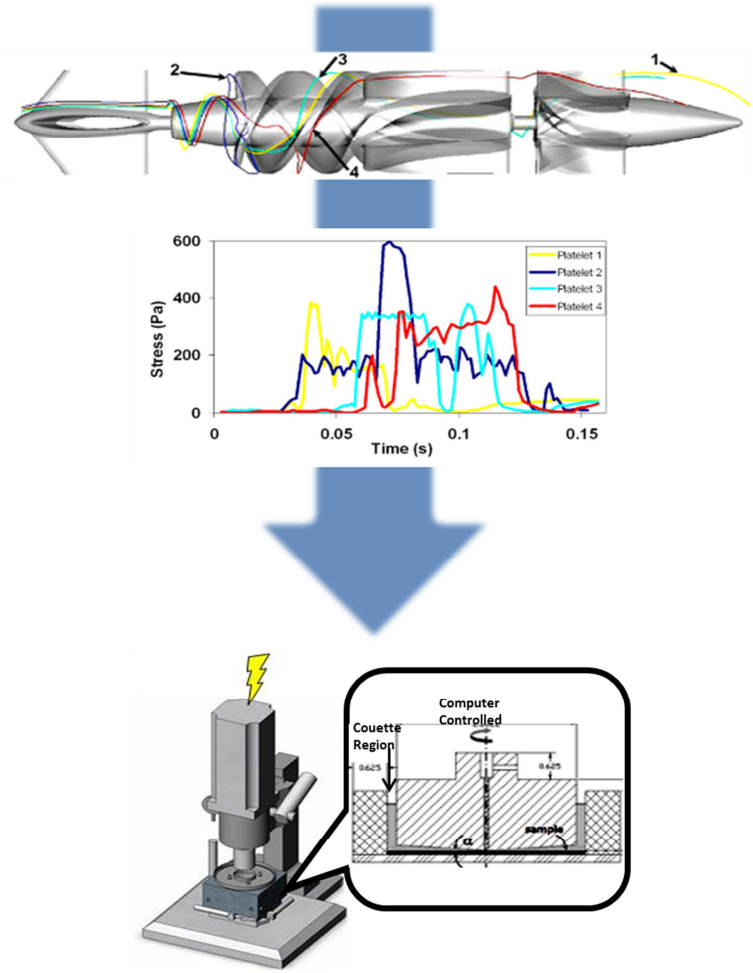

Figure 3.3 DTE Methodology**.** From *in silico* modelling to extraction of shear stress profiles tested with HSD: example with the Debakey VAD. Adapted from (Girdhar et al., 2012).

Flow path in the VAD is modeled with a high fidelity two-phase FSI (fluid-structure interaction) simulation, resolving all components of the stress tensor that are relevant to flow-induced thrombogenicity and subsequently tracking down and capturing the loading history (shear stress accumulation) of platelets in the flow field along trajectories that may drive them beyond the activation threshold. An example of platelet trajectories in the flow field of a VAD is shown at the top of Figure 3.3, while stress-loading waveforms corresponding to the four platelet trajectories, are shown in the center part of the Figure 3.3.

Cumulative stresses that may drive platelets beyond their activation threshold were calculated along multiple flow trajectories (thousands of simulated platelet) and collapsed into probability density functions (PDFs) (plotted in Figure 3.4) representing the device 'thrombogenic footprint' (Girdhar et al., 2012).

Such shearing profiles can be extracted from the PDF and tested in the HSD: platelet activity for these waveforms can thus be quantified with the PAS assay. Figure 3.4 displays the PDF

of the DeBakey VAD (red) and its optimized version, the Micromed HeartAssist 5 (HA5, blue). The yellow region represents the Hellums' threshold, that is the shear level over which platelets are considered activated (≈ 35 dyne s /cm^2) (Hellums et al., 1987). A difference between the two PDFs is clear; in particular, the Micromed HeartAssist 5 has a peak of the probability function close to the lower stress accumulation levels, thus indicating that this optimized device is characterized by an improved geometry with respect to the DeBakey VAD. The better characteristics of the former should lead to less invasive fluid dynamic conditions during the treatment with VAD.

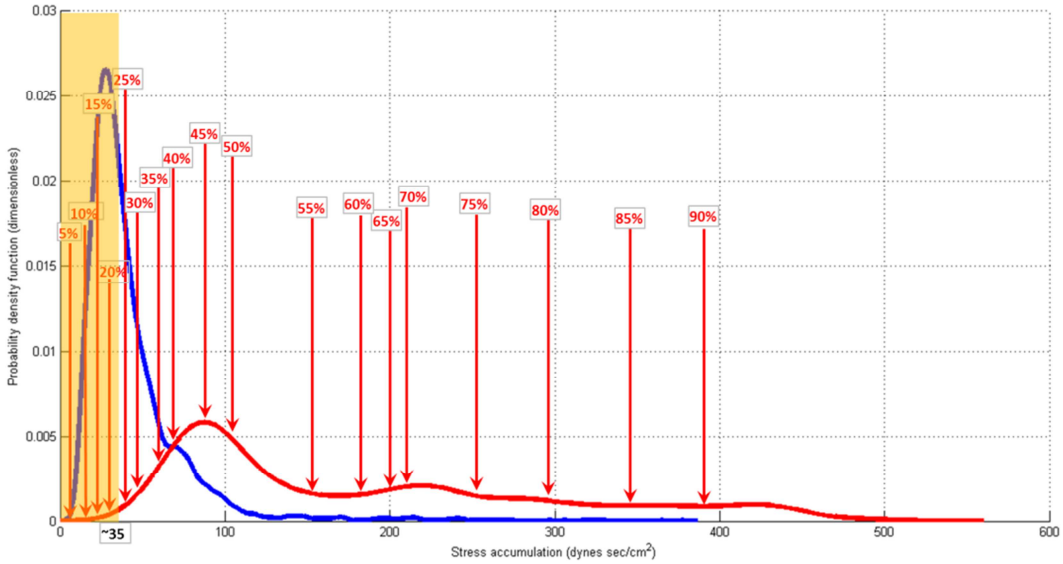

Figure 3.4 Probability Density Function (PDF) obtained from simulation within the DeBakey VAD (red) and HA5 (blue). Yellow region represent the shear stress interval considered harmless for platelets (Hellum's threshold). The red arrows indicate points in the PDF function that correspond to specific percentiles of Total Stress Accumulation characterizing the stress profiles within DeBakey VAD. Adapted from (Girdhar et al., 2012).

In the present study, we decided to investigate the behavior of several antiplatelet agents after subjecting GFP pre-treated with drugs to different shear stress profiles, either constant or dynamic, extracted from the PDF of the DeBakey VAD. For what concerns the constant conditions, platelets were subjected to 30 and 70 dyne/cm^2 for a total time of 10 min via the HSD. On the other hand, the used dynamic shearing waveforms were extracted from certain stress accumulation (SA) levels corresponding to different percentiles of the PDF. For the purpose of the study, two loading waveforms corresponding to the SA found at the 30th and 50th percentiles (Figure 3.5 - B) were implemented in the HSD. Hereinafter, these shear profiles will be dubbed Dynamic_30 and Dynamic_50 respectively. Such shear conditions were chosen because they were close to the peak in the PDF function (Figure 3.4), that was

reached at the 45th percentile. In this way we investigate shear conditions that most of the platelets experienced passing within the DeBakey VAD, according to the CFD simulations.

The limitations of the HSD didn't allow us to replicate ranges of shear stress peaks of 10^3 dyne/cm^2, as CFD simulations within VADs enlightened. Thus, a factor scale of 15 was used as trade off to reduce the shear stress obtained from CFD simulations to levels feasible for the machine but still interesting for the purpose of the study, which was to investigate the effect of antiplatelet agents under shear profiles similar to what platelets encounter *in vivo*. Despite this limitation, the HSD allowed to reproduce the extremely rapid dynamic shear stress profiles that platelets are subjected to while passing through VADs. The possibility to investigate platelet activation under such dynamic conditions results fundamental. In fact, studies have stressed out as platelets are more sensitive to dynamic shearing profiles compared to constant ones (Sheriff J, Bluestein D et al., 2010; Sheriff et al., 2013).

Since the exposure time characterizing such extracted profiles was in the order of few milliseconds, the waveforms were then looped within HSD to match the total exposure time experienced by platelets during experiments under constant shear stress conditions (10 min). An example of the tested shear profiles is reported in Figure 3.5.

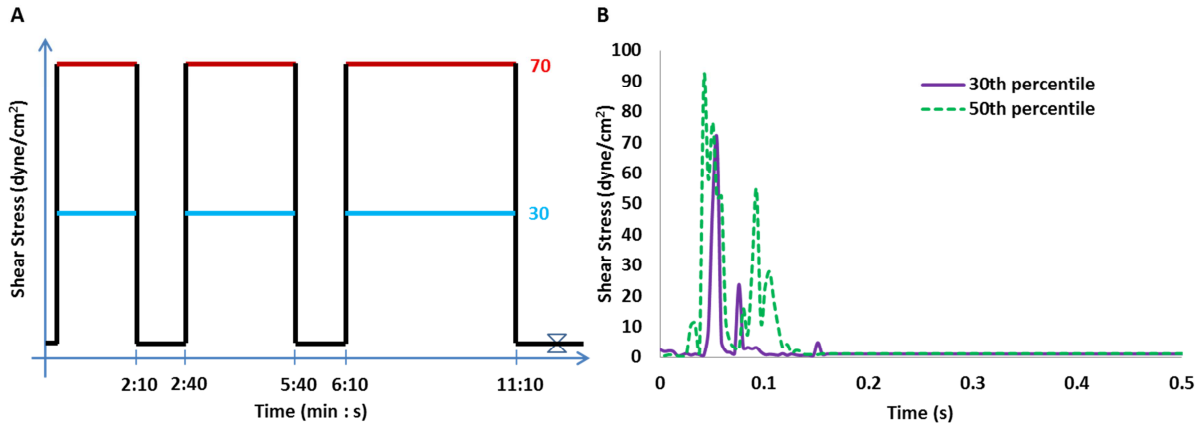

Figure 3.5 Constant and dynamic shear stress waveforms. A. Shear stress patterns during the whole experiment under constant conditions. B. Dynamic shear stress waveforms representing a single cycle of generic Debakey VAD. This cycle was looped within the HSD for the entire duration of the experiment (10 min).

After incubation with antiplatelet agents, platelet samples were thus subjected to the shear stress conditions via HSD. For each sample, a control experiment consisting of platelets not treated with drugs and subjected to the same shear conditions was performed.

500 µl of solution were extracted from the HSD after 2, 5 and 10 min of exposure to shear stress and prepared for the Platelet Activity State (PAS) assay (Paragraph 3.3.5).

Each experiment, consisting in exposing a sample of platelets (4 ml) incubated with one drug to shear stress via HSD, needed 20 min to be performed. Assuming that a gradual decay of platelet vitality occurs few hours after GFP collection (5 to 6 hours), a maximum of 15 experiments were conducted each day.

3.3.5 The Platelet Activity State (PAS) Assay

Platelet activity state (PAS) was measured using a modified prothrombinase method developed at Stony Brook University (D. Bluestein and J. Jesty) (Jesty et al., 1999). This method is particularly well suited for identifying activation due to physical forces, such as fluid shear or turbulence, encountered by the blood passing through cardiac devices. This assay utilizes a novel stoichiometric relationship identified in a clinical coagulopathy known as “Prothrombin Quick disorder”. Although thrombin generation is a powerful marker of platelet activation in response to several biochemical and mechanical agonists, the produced thrombin has a positive feedback response on platelet activation. In the PAS assay, there is a linear relationship between platelet activity and the inducer, a modified prothrombin (Ac-FIIa) which does not feedback on the factor Xa complex to further activate additional platelets or convert fibrinogen to fibrin. In the assay, the research group in Stony Brook mimics Prothrombic Quick disorder by acetylating prothrombin, thus creating the reagent for this assay. The removal of the positive feedback activation by thrombin is essential for measuring platelet activation, which results in “linear” thrombin generation such that a one-to-one correspondence is established between the inducer (agonist) and platelet activity. Such modification to the thrombin generation cascade is summarized in Figure 3.6.

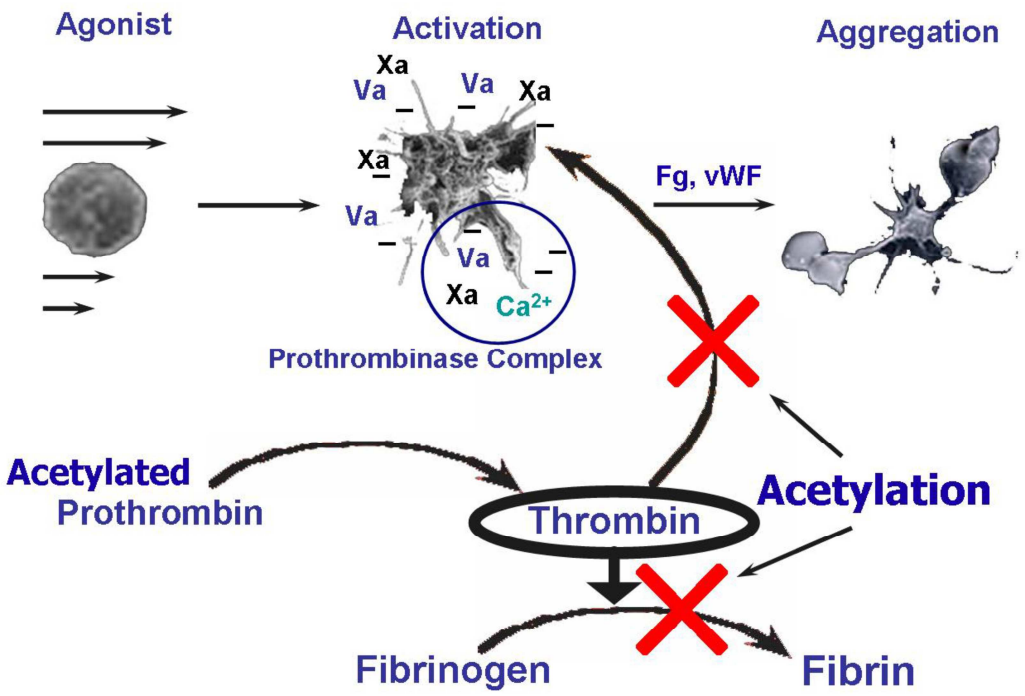

Figure 3.6 The Platelet Activity State (PAS) assay. Fibrin formation, as well as the positive thrombin feedback that further activates platelets, are prevented by the prothrombin acetylation. This allows for a direct correlation between the agonist, in this case shear stress, and PAS, measured by thrombin generation (Sheriff J, 2010).

In order to obtain the acetylated prothrombin (Ac-FIIa), 10 µM prothrombin was treated for 20 min at room temperature in a solution of 100 mM $NaHCO_3$ + 5 mM $CaCl_2$ with 3 mM sulfo-N-succinimidyl acetate (Jesty et al., 2003). Platelet samples obtained during the experiments were taken at different time points from the experimental setup and assayed using the PAS assay. For an initial platelet concentration of 20,000/µl, PAS was measured as in the following. A tube of 100 µl, containing 5,000 platelets/µl, 100 pM Factor Xa (FXa), 5 mM Ca_2^+, and 200 nM Ac-FIIa was incubated for 10 min at 37°C. A sample of 10 µl was taken from the incubated tube and assayed for thrombin generation by using 0.3 mM Chromozym TH (CH-TH, Tosyl-Gly-Pro-Arg-4-nitranilide acetate, Roche Diagnostics, Indianapolis, IN) in a 96-well microplate reader (Vmax, Molecular Devices, Sunnyvale, CA), at 25°C. Thrombin generation was analyzed over 7 min at an absorbance wavelength of 405 nM (Sheriff J, 2010). The changes in absorbance/min were computed at each time point and normalized with respect to the thrombin generation rate of fully activated platelets obtained by sonication (75 W for 10 s, Branson Sonifier 150 with microprobe, Branson, MO). Thus, all PAS values were expressed as a fraction of the maximum thrombin-generating capacity, with a maximum of 1.0. This assay allows for a 1:1 correlation between the applied shear stress and thrombin generation, and activation changes as low as 0.1 % can be detected.

Hepes-buffered saline (HBS) with 0.1% BSA (HBS:BSA), at a physiological pH of 7.4, was used as buffer solution for the tubes in which platelets were incubated with Ac-FIIa, Ca_2^+, and FXa in the concentrations reported above. For the microplate wells, Chromozym TH, dissolved in 0.15 M NaCl, adjusted to a concentration of 1 mM, was buffered with HBS:BSA + 5 mM ethylenediaminetetraacetic acid (EDTA) at a pH of 7.4 (Sheriff J, 2010).

3.3.6 Statistical analysis

Statistical tests were performed using SigmaPlot (Systat Software Inc). Shapiro Normality test was run for all population of data before significance tests. Samples treated with antiplatelet agents were compared with their controls after 10 min of stimulation. Either parametric or non-parametric (Kruskal-Wallis) one-way ANOVA were performed depending on the distribution of the data analyzed. Single comparison t-test was used in two-population comparisons when possible, while Mann-Whitney Rank Sum Tests were performed when non-parametric analysis between two groups were needed. Differences were considered significant if $p < 0.05$.

3.4 Results

3.4.1 Characterization of shear-mediated platelet activation

Platelets were first exposed to experimental shear stress profiles without any antiplatelet drug, in order to characterize the shear-mediated level of platelet activation and define the experimental conditions to test with the different drugs. Platelets were subjected to 10, 30, 50 and 70 dyne/cm^2 for a total time of 10 min. Samples (500 µl) were taken at 2, 5 and 10 min using a 1 ml syringe appropriately connected to the HSD. Concerning the dynamic waveforms, different shearing profiles corresponding to SA levels at the 30th and 50th percentiles of the DeBakey VAD PDF were uploaded and repeated cyclically for a total of 1100 loops, equal to 10 min inside the HSD. The results obtained, normalized with respect to fully activated platelets (paragraph 3.3.5), are represented in Figure 3.7.

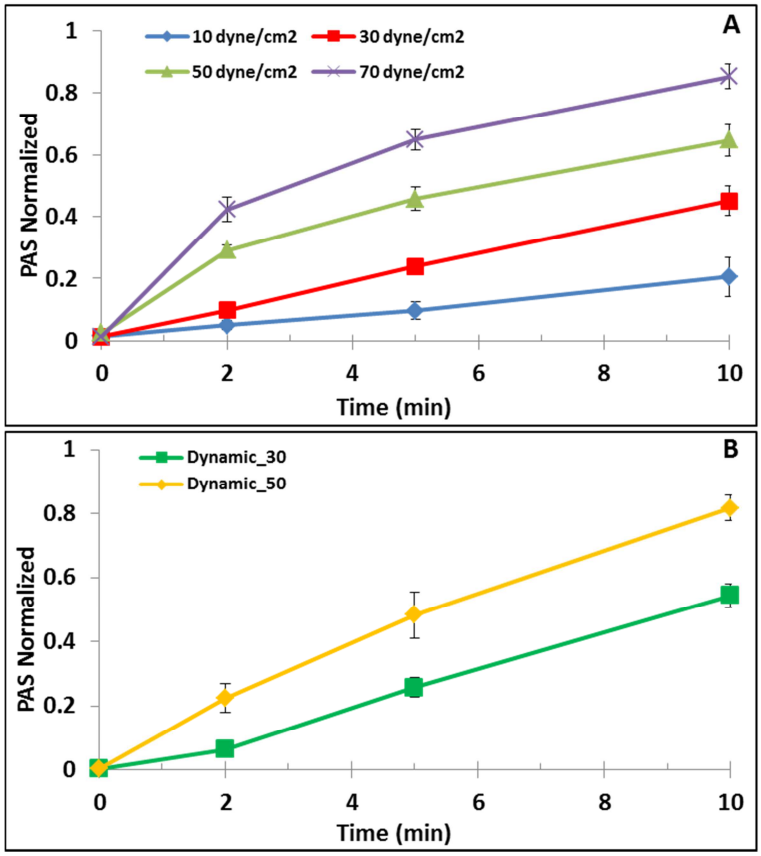

Figure 3.7 Platelets activation under constant (A) and dynamic (B) shear stress conditions via HSD. N Expt = 7+ from at least 7 different donors. Data are reported as mean ± SEM.

As evident in Figure 3.7, platelet activation depends both on the level of shear and on the duration of the stimulation. In fact, platelet activation level after 10 min of experiment span from 20% to 84% at 10 dyne/cm^2 or at 70 dyne/cm^2, with respect to fully activated platelets.

In accordance with the results obtained we decided to use just the 30 and 70 dyne/cm^2 shear stress levels in the constant shear stress configuration. Indeed, it was not relevant to keep all four shear profiles, even because no significant new data would emerge from a such detailed experimental scenario. Instead, regarding the dynamic experiments, both the loading waveforms extracted at the 30th and 50th percentiles of the Debakey VAD PDF were utilized for testing the antiplatelet agents. All data presented in the present chapter are represented as mean ± SEM, unless otherwise stated.

3.4.2 Effect of COX Inhibitors on shear-mediated platelet activation

The concentration of aspirin (ASA) chosen for the experiments was 25 and 125 µM in 4 ml of platelet sample, thus corresponding to clinical use dosage of 81 mg/day or 325 mg/day (Ensor et al., 2010). Results obtained are described in Figure 3.8, where A-B correspond to 30 and 70 dyne/cm^2 and C-D to the dynamic waveforms extracted from simulation in the Debakey VAD (Dynamic_30 and Dynamic_50). For each ASA-treated platelet sample, a paired control experiment with untreated platelets subjected to the same shear conditions was performed on the same day.

The number of experiments performed differs among shear conditions (range 10-38), with blood samples from at least 6 donors.

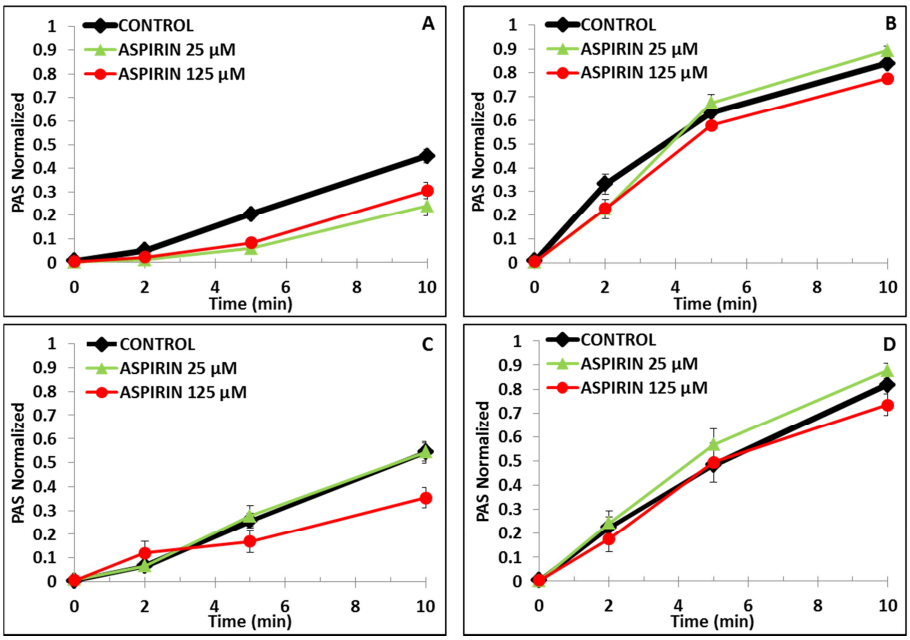

Figure 3.8. The effect of aspirin on shear-mediated platelet activation. The effect of aspirin (25 – 125 µM) was investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 10+ from at least 6 different donors. Data are reported as mean ± SEM.

The effect of ASA on platelets activation was different between shear stress conditions. In fact, from the statistical analysis of constant condition experiments, it emerged that ASA offers a protection effect at low shear, i.e. 30 dyne/cm^2 ($p < 0.004$ compared to control for both concentration), but no significant effect was achieved at 70 dyne/cm^2 ($p > 0.5$). A similar behavior was found in the dynamic conditions, where aspirin showed a slightly lower reduction at Dynamic_30 stimulation ($p < 0.05$ only for ASA 125 µM compared to control), with no effect for the most invasive condition.

3.4.3 Effect of phosphodiesterase inhibitors on shear-mediated platelet activation

Cyclic adenosine phosphodiesterases (PDEs) limit the intracellular levels of cyclic nucleotides, thus regulating platelet function and activation. Several nonselective or isoenzyme-selective PDE inhibitors have been developed, and some of them are currently used in clinic as antiplatelet agents (Gresele et al., 2011). The phosphodiesterase inhibitors investigated in the present study were dipyridamole, cilostazol and pentoxifylline. The number of experiments performed at each concentration of drugs tested in this section were at least 6, with blood samples obtained from at least 3 different donors.

Dipyridamole acts as an inhibitor of PDE5, thus increasing intraplatelet cGMP and in turn leading to platelet activation inhibition. This drug was tested at three concentrations (5, 10, 25 µM). The results are reported in Figure 3.9.

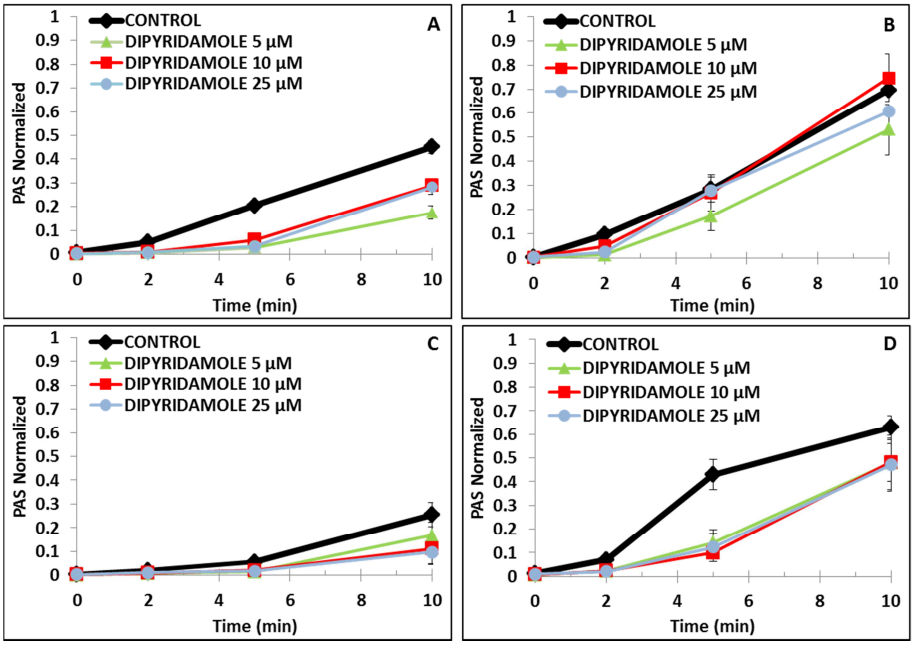

Figure 3.9. The effect of dipyridamole on shear-mediated platelet activation. The effect of dipyridamole (5, 10, 25 µM) was investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 6+ from at least 3 different donors. Data are reported as mean ± SEM.

The protective effect provided by dipyridamole was concentration dependent only after exposure to 30 dyne/cm^2 and decreased at higher shear stress condition. Data obtained at 30 dyne/cm^2 after 10 min of shear exposure showed a significant reduction in the activation level of platelets treated with the drug with respect to the control ($p < 0.05$). On the other hand, in the other tested conditions dipyridamole presented no consistent protective action in time, with low differences compared to non-treated samples that cannot be considered relevant to the study.

Cilostazol has the capacity of diminish intracellular calcium and can cause smooth muscle cell relaxation and platelet activation inhibition (Gresele et al., 2011). Results obtained after incubating platelets with cilostazol at a concentration of 50 µM are illustrated in Figure 3.10.

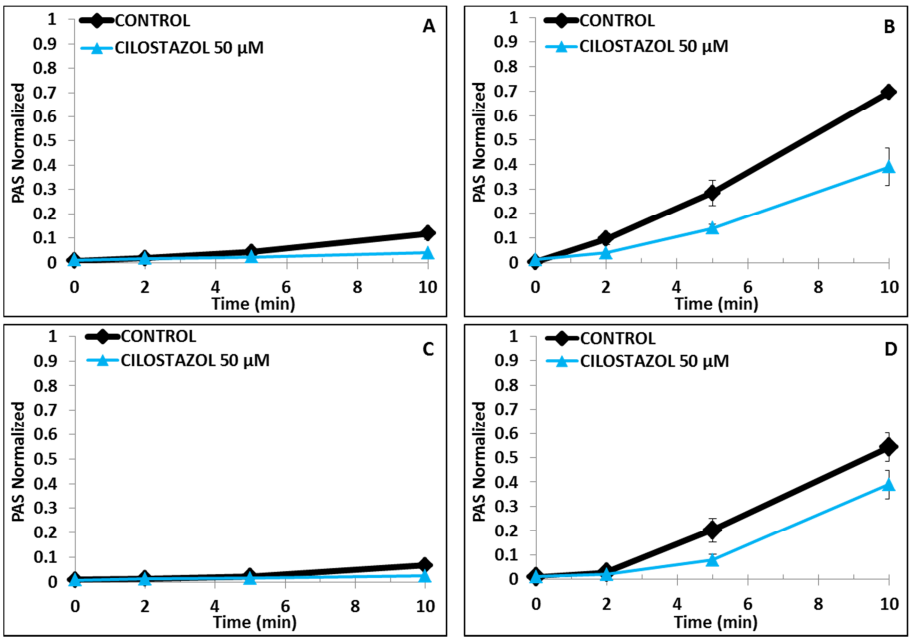

Figure 3.10. The effect of cilostazol on shear-mediated platelet activation. The effect of cilostazol (50 µM) was investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 6+ from at least 3 different donors. Data are reported as mean ± SEM.

A clear difference emerged for low shear stress conditions between the level of platelet activation for the control group used for cilostazol after 10 min compared to the control group obtained for the tests of previous drugs (0.12 ± 0.02 vs 0.45 ± 0.03 for 30 dyne/cm^2 , 0.07 ± 0.01 vs 0.54 ± 0.04 for the Dynamic_30).

This discrepancy was due to the use of a less sensitive acetylated prothrombin during the PAS assay, thus in turn leading to a reduced thrombin release detection for both samples treated with the drug and their control.

Despite this difference, data were analyzed as percentages of reduction provided by the antiplatelet agents compared to their corresponding non-treated samples. In this scenario, cilostazol showed a protection effect in both the constant conditions, with a reduction of 66% provided by the drug at 10 min for the 30 dyne/cm^2 ($p < 0.001$) and 44% for 70 dyne/cm^2 ($p < 0.002$). Concerning the dynamic condition, the drug provided a reduction of 63% compared to control at Dynamic_30, with a $p < 0.001$, while no significant protection effect was obtained at Dynamic_50 ($p = 0.09$).

The last phosphodiesterase (PDE) receptors inhibitor studied was pentoxifylline. It is a non-selective PDE inhibitor that reduces whole blood viscosity and improves erythrocyte deformability. Samples treated with pentoxifylline were tested after shear exposure via the HSD. The results are represented in Figure 3.11.

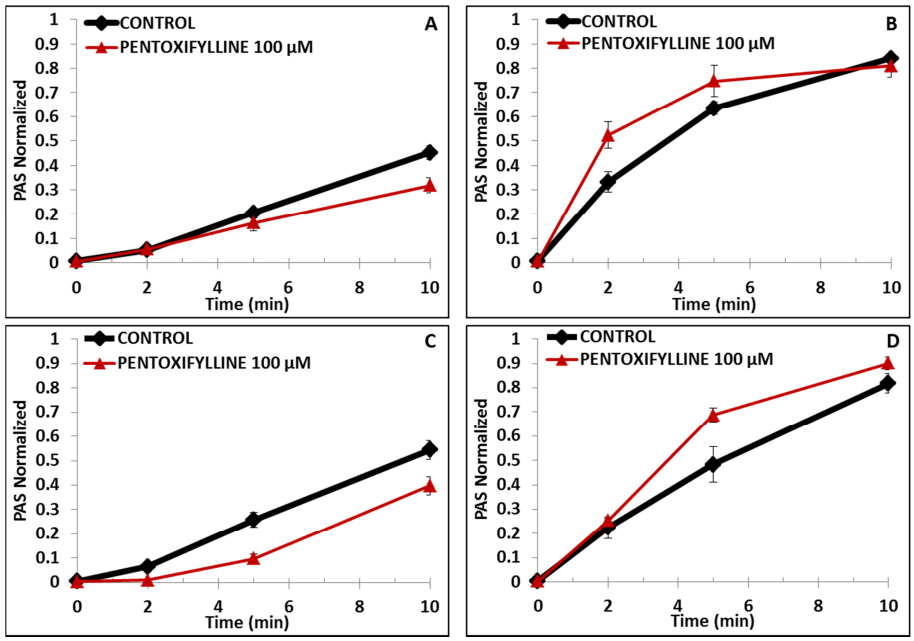

Figure 3.11. The effect of pentoxifylline on shear-mediated platelet activation. The effect of pentoxifylline (100 µM) was investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 6+ from at least 3 different donors. Data are reported as mean ± SEM.

Data indicated that pentoxifylline only slightly reduced platelet activation at low shear condition, with a maximum significant inhibition of 30%, obtained for 30 dyne/cm^2 ($p < 0.027$). Similar values were reached after subjecting platelets to the Dynamic_30, with consistent reduction and $p < 0.03$ compared to control. An opposite trend was instead achieved at high shear stress condition (70 dyne/cm^2, Dynamic_50), with a slight increase in platelet activity state of the samples treated with the drug compared to the control group, thus indicating at least no protection effect provided by the drug at high shear conditions.

3.4.4 Effect of GPIIb-IIIa inhibitors on shear-mediated platelet activation

Platelet membrane activated receptor glycoprotein GPIIb-IIIa can bind fibrinogen, starting the final common pathway of platelet aggregation. Since the GPIIb-IIIa receptor is the most important integrin to be involved in platelet aggregation, the development of GPIIb-IIIa antagonists like eptifibatide (trademarked as Integrilin) has become an attractive strategy for antiplatelet therapy with an expected strong and specific effect. In this study, we deemed opportune to study the effect of eptifibatide on shear mediated platelet activation (Figure 3.12).

Also in this case, the minimum number of experiments performed was 6, with blood samples obtained from at least 4 different donors.

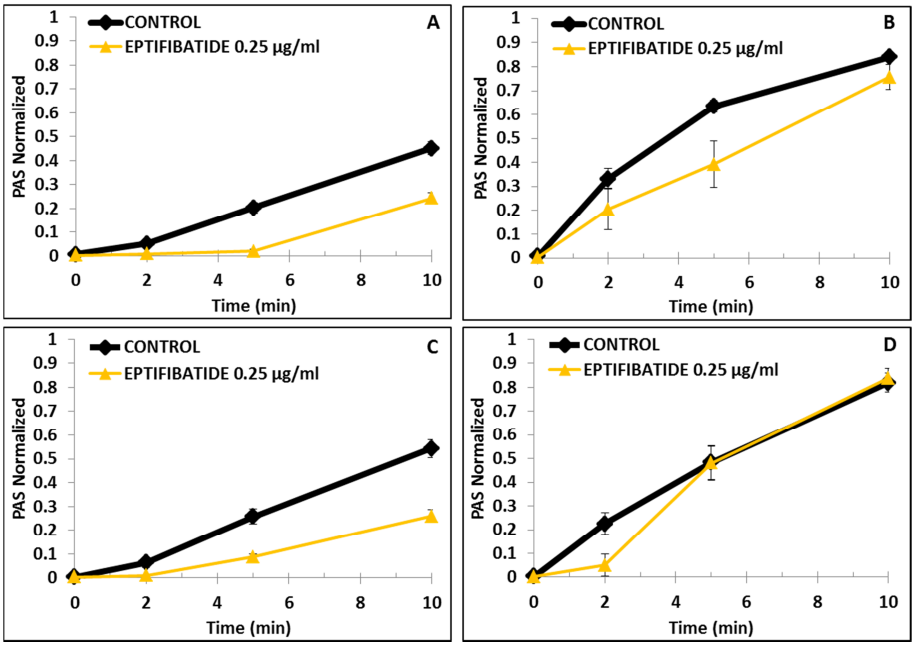

Figure 3.12. The effect of eptifibatide on shear-mediated platelet activation. The effect of eptifibatide (0.25 µg/ml) was investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 6+ from at least 3 different donors. Data are reported as mean ± SEM.

The concentration of eptifibatide tested in the present study was 0.25 µg/ml. This amount was considered correct for the investigation, based on results obtained from an experiment in which different concentration of eptifibatide were tested to identify the one that may be both effective and non-toxic for platelets.

As seen with several antiplatelet agents, eptifibatide showed a remarkable platelet protection when subjected to low shear stimulation, with an overall reduction of platelet activation of 50% after 10 min for both the 30 dyne/cm^2 and the Dynamic_30 ($p < 0.003$).

The protection effect provided by the drug resulted instead overwhelmed by the physical forces at higher shear stress conditions.

3.4.5 Effect of ADP-receptor blockers on shear-mediated platelet activation

Ticagrelor reversibly interact with the platelet $P2Y_{12}$ ADP-receptor to prevent signal transduction and platelet activation. The effect of ticagrelor was studied at two different concentration, 10 and 100 µM (Tomizawa et al., 2013) in the same experimental condition described for the investigation of the previous agents. The obtained results are represented in Figure 3.13.

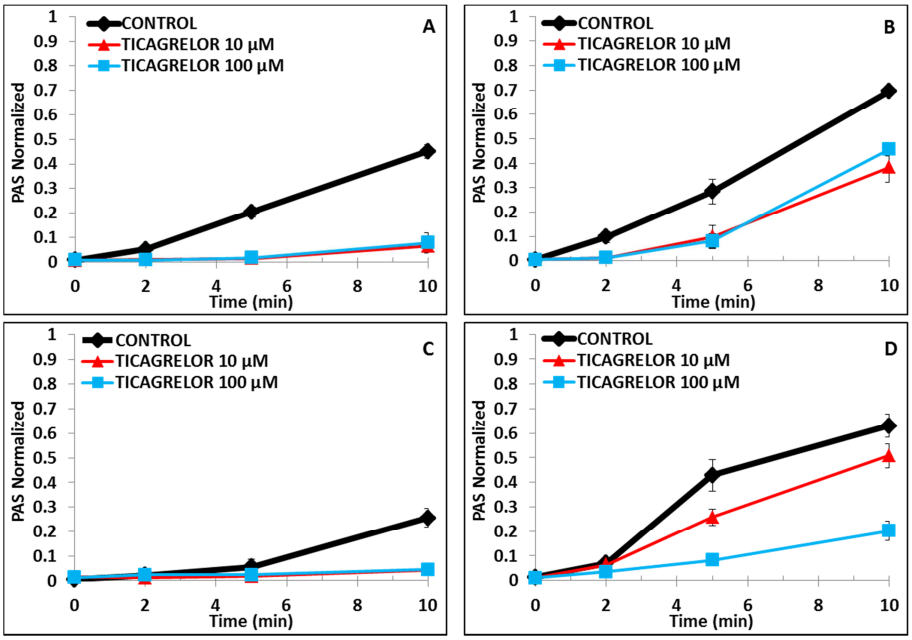

Figure 3.13. The effect of ticagrelor on shear-mediated platelet activation. The effect of ticagrelor (10 – 100 µM) was investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 6+ from at least 3 different donors. Data are reported as mean ± SEM.

Ticagrelor showed a stronger protection action compared to all antiplatelet drugs tested in the present study. In fact, the reduction provided by this drug appeared consistent for all shear profiles. In constant condition, both concentrations inhibit platelet activation. At 30 dyne/cm^2, the reduction compared to control after 10 min of shear exposure was > 80% (Figure 3.15), with $p < 0.05$. Different was the response of platelets subjected to 70 dyne/cm^2, with an overall reduction of activation provided by ticagrelor of 40%, still statistically significant for the study ($p < 0.05$). The results obtained with the dynamic conditions showed a strong response provided by both concentrations of the drug for Dynamic_30 (> 80% reduction, $p < 0.05$). At higher dynamic shear stress condition, ticagrelor showed a consistent protection

action when used at high concentration (100 µM), with a reduction with respect to control even stronger compared to the effect obtained with the constant counterpart stimuli (68% vs 40% reduction at 10 min), with $p < 0.05$. On the other hand, the tests performed using ticagrelor at a concentration of 10 µM found no significant platelets inhibition with respect to control condition ($p > 0.05$), despite a mean reduction of 19% at 10 min after shear stress exposure.

3.4.6 Effect of aspirin in combination with other drugs on shear-mediated platelet activation

The use of aspirin in combination with other antiplatelet agents has been studied by several research groups (Cattaneo M., 2004; Taylor et al., 1992; Cooke et al., 2006; Gresele et al., 2011; Diener et al., 1996; Lentz et al., 2000; Verro et al., 2008; Adams et al., 2008; Ratnatunga C.P., 1992). This combined approach has been introduced in clinical management of MCS patients. With the aim of investigating the effectiveness of these therapies, we studied the level of platelet activation inhibition provided by low concentration aspirin (25 µM) in combination with PDEs inhibitors like dipyridamole (5µM) (Gresele et al., 2011) and pentoxifylline (100 µM) (Ueno et al., 2011) or GP-IIb-IIIa inhibitor like eptifibatide (0.25 µg/ml) (Nannizzi-Alaimo et al., 2003). Finally, we tested the combination of aspirin + pentoxifylline + eptifibatide on platelet reactivity (Figure 3.14).

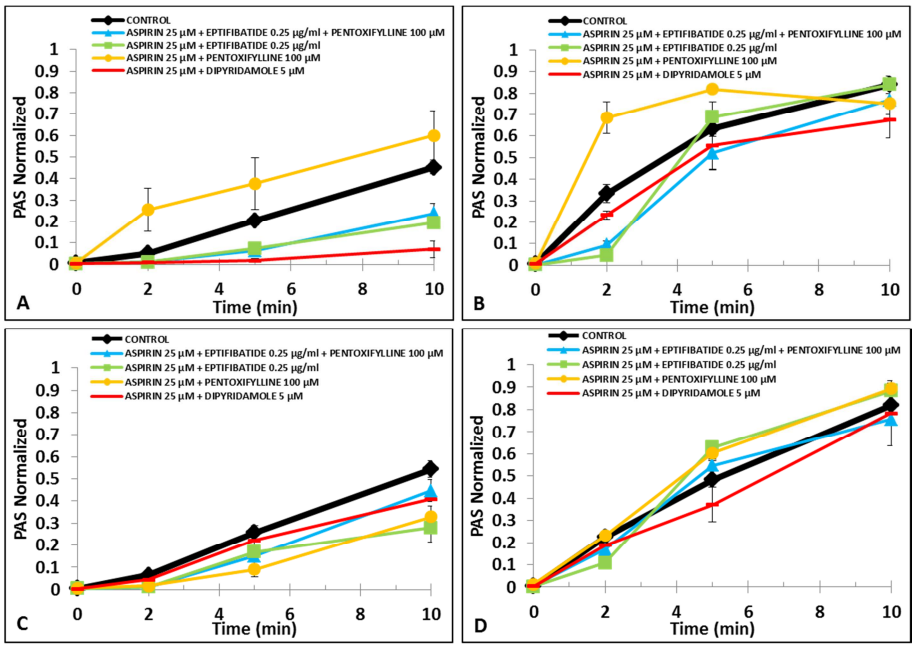

Figure 3.14. The effect of aspirin in combination with other antiplatelet agents on shear-mediated platelet activation. The effect of aspirin (25 µM) in combination with eptifibatide (0.25 µg/ml), pentoxifylline (100 µM), dipyridamole (5 µM), or eptifibatide + pentoxifylline were investigated after exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C), Dynamic_50 (D). N Expt = 6+ from at least 3 different donors. Data are reported as mean ± SEM.

The results showed a potentiated effect offered by some drugs in combination with low dose aspirin, but only for the 30 dyne/cm^2 and the Dynamic_30 conditions. In fact, platelets protection was increased when aspirin was used in combination with dipyridamole or eptifibatide, with a percent reduction after 10 min at 30 dyne/cm^2 equal to 84% and 57% respectively, versus a reduction of 47% provided by aspirin alone. Differently, the use of pentoxifylline in combination with aspirin showed a potentiation effect of shear-mediated platelet activation. The behavior of platelets treated with different combination of drugs and subjected to Dynamic_30 was similar to constant conditions. Unlike the effect of aspirin alone at a concentration on 25 µM, which provided no platelet protection at 10 min after shear exposure (3.4.2), the combination with other agents provided a reduction of platelet activation. 42%, 49% and 40% reduction (Figure 3.15) were indeed obtained for ASA combined with dipyridamole, eptifibatide and pentoxifylline respectively, with $p < 0.005$. The use of aspirin in combination with both eptifibatide and pentoxifylline demonstrate a mean reduction of platelet activation of 18% compared to control, but no statistical differences was found among the two populations of data ($p = 0,1$). No protection was provided by antiplatelet agents for high shear stress exposure conditions. This finding supports the theory that there is a level of shear-induced platelet activation that cannot be modified by the effect of antiplatelet drugs, because of the inadequacy of their mechanisms of protection after exposure to elevated mechanical stimuli.

3.4.7 Percent reduction of platelet activation provided by antiplatelet agents after 10 min of exposure at different shear stress profiles

As emerged from the results, the protection effect provided by the antiplatelet agents was strongly dependent on the shear stress level to which platelets were exposed. In an attempt to analyze the behavior of all the agents tested, the percentage of platelet activation reduction was calculated for all kinds of samples treated with drugs compared to control, after being subjected to the four shear stress profiles described in Paragraph 3.3.4. The graphical representation of these data is reported in Figure 3.15.

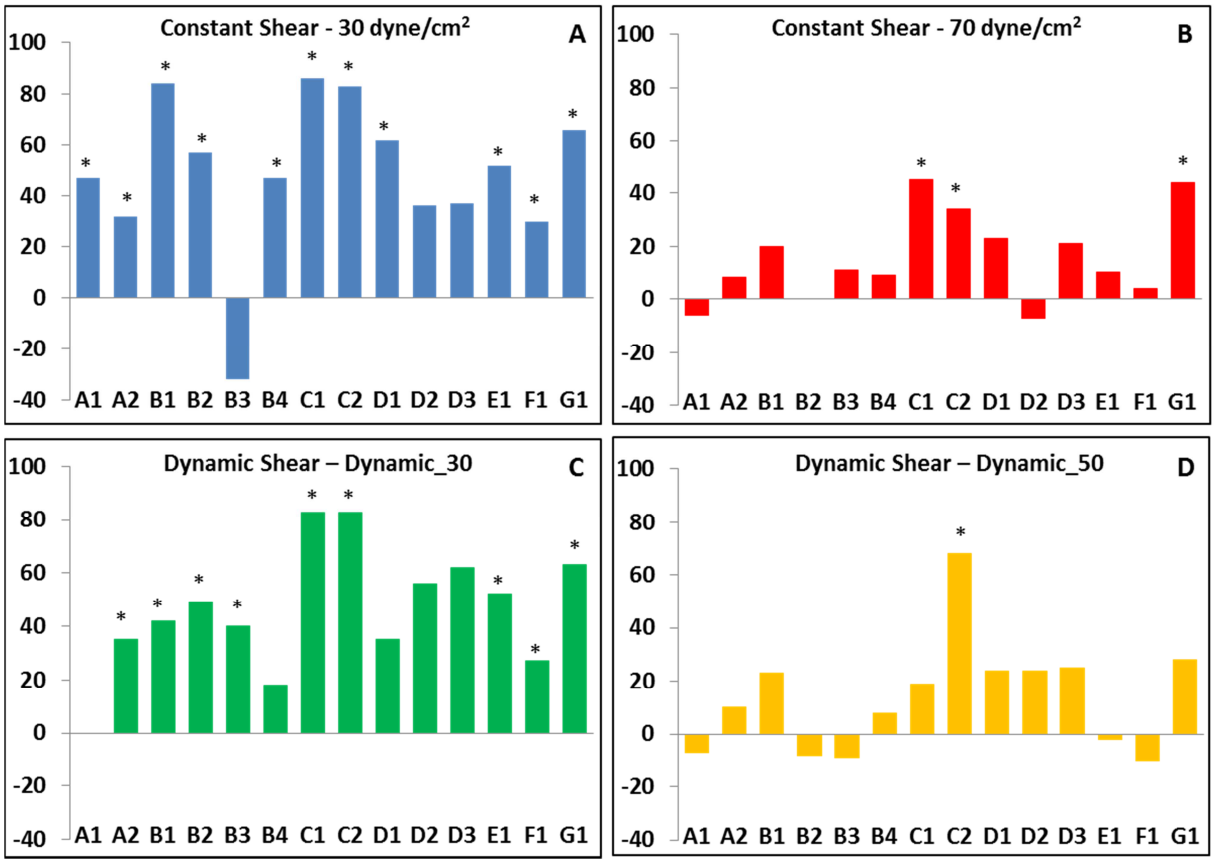

Figure 3.15. Mean % of platelet activation reduction provided by different antiplatelet agents (A1-G1) tested after 10 min exposure to 30 dyne/cm^2 (A), 70 dyne/cm^2 (B), Dynamic_30 (C) and Dynamic_50 (D). % reduction are intended compared to control group. Negative numbers indicate an actual increase of platelet activation after treatment with the drug: see text for discussion of such specific cases. (* $p < 0.05$). Antiplatelet agents: A: aspirin alone (A1 - 25 µM, A2 - 125 µM); B:aspirin in combination with other drugs (B1- ASA 25 µM + dipyridamole 5 µM, B2 - ASA 25 µM + eptifibatide 0.25 µg/ml, B3- ASA 25 µM + pentoxifylline 100 µM, B4- ASA 25 µM + eptifibatide 0.25 µg/ml + pentoxifylline 100 µM); C: ticagrelor (C1 - ticagrelor 10 µM, C2 - ticagrelor 100 µM); D: dipyridamole (D1 - dipyridamole 5 µM, D2 - dipyridamole 10 µM, D3 - dipyridamole 25 µM); E1: eptifibatide 0.25 µg/ml; F1: pentoxifylline 100 µM; G1: cilostazol 50 µM.

As emerged from Figure 3.15, antiplatelet agents provided a stronger protection to platelets subjected to lower shear profiles than to higher profiles (70 dyne/cm^2 or Dynamic_50). In particular, at 30 dyne/cm^2 the majority of the tested agents showed a protection effect, with a mean reduction of 55%. The same trend was found with platelets subjected to the Dynamic_30 condition, where most drugs provided a significant inhibition of platelet activation and a mean reduction of 50% compared to control. Platelets treated with aspirin in combination with pentoxifylline actually displayed an abnormal behavior, consisting in an increase in platelet activation. This was probably due to errors in the dosage chosen for the experiments performed.

Results obtained when platelets were tested at higher shear stress conditions showed a different trend. In fact, poor protection was ensured by antiplatelet agents at 70 dyne/cm^2. The only drugs that were found to be effective also at this shear stress level were ticagrelor and

cilostazol, which ensured a statistically significant reduction of platelet activation of 40% ($p < 0.05$). Even worse was the effect granted by antiplatelet agents after subjecting platelets to Dynamic_50 shearing profile. Indeed, in this case, the only condition that seems to provide a certain inhibition of platelet reactivity was the treatment with ticagrelor, at a concentration of 100 µM.

3.5 Discussion

In the present study we compared the effect of different antiplatelet agents after exposure to both constant and dynamic shearing profiles, with the goal of understanding the behavior of these drugs in protecting shear-activated platelets.

To date, current antithrombotic therapies, designed primarily to modulate biochemical pathways of platelet activation, have been poorly studied in combination with physical forces, such as shear profiles that mimic fluid dynamic conditions inside VADs. To the best of our knowledge, only few research groups have tried to subject platelets treated with currently used antiplatelet drugs to physical stimuli for assessing the ability of those agents to protect platelets (Moake et al., 1988; Minami et al., 1997; Sheriff et al., 2014; Tomizawa et al., 2013). This kind of investigation resulted thus mandatory to ensure a correct characterization of the drugs' ability to protect platelets from mechanical stimuli.

First, platelets were subjected to physical forces for a total exposure time of 10 min without drugs. The results showed that platelet activation depends both on the level of shear and on the duration of the stimulation, reaching a level up to 84% of maximum activation obtained via sonication at 75 W for 10 s.

After this preliminary characterization of shear-induced platelet activation, we subjected platelets incubated with several antiplatelet agents (Table 3.1) to different shear stress profiles, both constant and dynamic, via the HSD.

As a COX-inhibitor, aspirin was studied at two different concentrations of interest, i.e. 25 µM and 125 µM (Ensor et al., 2010). The protective effect of aspirin was found only after exposure to low shear stress profiles (30 dyne/cm^2), with poor platelet activation reduction at higher shear waveforms.

Another category of antiplatelet agents investigated was the phosphodiesterase receptor inhibitors. Among them the drugs analyzed were dipyridamole, cilostazol and pentoxifylline (Kullmann et al., 1993; Meester et al., 1998; Kariyazono et al., 2001). A study performed by Weithmann (1980) assessed that pentoxifylline, as well as dipyridamole, should manifest a stronger platelet protection effect *in vivo* compared to *in vitro* condition because their platelet inhibitory effect are enhanced by Prostacyclin (PGI_2), a lipid molecule mainly presents in the blood vessel walls. Platelets reactivity once incubated with dipyridamole and subjected to shear stress in our *in vitro* set up was concentration dependent only after exposure to 30 dyne/cm^2 and decreased at higher shear stress conditions. Data obtained after 10 min of shear exposure at 30 dyne/cm^2 showed a significant reduction in the activation level of platelets

treated with the drug ($p < 0.05$). On the other hand, dipyridamole presented no consistent protective action in time at both 70 dyne/cm^2 and Dinamic_50, with low differences compared to non-treated samples that cannot be considered relevant to the study. A similar effect was provided by pentoxifylline, that only at low shear stress seemed to protect platelets. Different was the behavior of cilostazol. The latter indeed showed a protection effect in both the constant conditions, with a reduction of 66% provided by the drug at 10 min for the 30 dyne/cm^2 ($p < 0.001$) and 44% for 70 dyne/cm^2 ($p < 0.002$). Concerning the dynamic condition, cilostazol provided a reduction of 63% compared to control at Dynamic_30, while no significant protection effect was obtained at Dynamic_50 ($p = 0.09$). The vasodilatory and antiplatelet actions of cilostazol are due mainly to the inhibition of phosphodiesterase 3 (PDE3) and subsequent elevation of intracellular cAMP levels. Recent preclinical studies have demonstrated that cilostazol also possesses the ability to inhibit adenosine (ADO) uptake, a property that may distinguish it from other PDE3 inhibitors (Liu Y. et al., 2001). Adenosine, as well as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) plays an important role in the biochemical process. Adenosine also plays a role in regulation of blood flow to various organs through vasodilation. More important, ADP is considered a key factor in the starting phase of platelet activation. The ability action of cilostazol to inhibit both PDE3 and ADO may be the reason of its stronger protection of platelets exposed to shear stress.

As mentioned in paragraph 3.2, GPIIb-IIIa receptor is one of the most important integrin to be involved in platelet aggregation since it enables to bind fibrinogen, a key element in blood clot formation. In the present study, eptifibatide showed a great platelet protection when subjected to low shear stimulation, with an overall reduction of platelet activation of 50% after 10 min for both the 30 dyne/cm^2 and the Dynamic_30 ($p < 0.003$). Nonetheless, the protection effect provided by eptifibatide resulted overwhelmed by the physical forces at higher shear stress conditions, thus confirming the idea that antiplatelet agents poorly protect platelet from shear-mediated activation. This behavior is due to the process of development of new drugs, in which principal attention is focused on providing chemical inhibition of platelet activation instead of protection from external physical forces, like the ones experienced passing through cardiac assist devices as VADs or TAH.

Aspirin alone provided a limited protection to platelets subjected to all kind of shear stress in the present investigation. In the last years, a combined approach consisting of coupling low dosage of aspirin in combination with other antiplatelet agents has been introduced in clinical management of MCS patients (Ensor et al., 2010). We thus studied

aspirin in combination with dipyridamole, pentoxifylline and eptifibatide. Results showed a partial decrease in platelet activation but only at low shear stresses. Moreover, when aspirin was combined with pentoxifylline, PAS assays showed a seemingly paradoxical behavior, i.e. a potentiation effect of shear-mediated platelet activation. This finding may be explained in different ways. One hypothesis is that the drug concentration chosen was already toxic for the cells, while an alternative is that the mechanism of action provided by the two drugs may be antagonist for what concern platelets protection. It emerged therefore how delicate is the process of developing complicated antithrombotic therapies. The latter combine several drugs with the goal of potentiating the effect on platelet activation. Besides these treatments are mandatory in certain cases, a great attention should be paid to avoid the occurrence of side effects harmful for platelets.

The only drug that provided an effective reduction after exposure to every shear stress profiles tested in our work was ticagrelor. The latter reversibly interacts with the platelet $P2Y_{12}$ ADP-receptor to prevent signal transduction and platelet activation. Ticagrelor showed a consistent protection action when used at high concentration (100 µM), with a reduction with respect to control after exposure to a dynamic waveform even stronger compared to the effect obtained with the constant counterpart stimuli (68% vs 40% reduction at 10 min), with $p < 0.05$.

The antiplatelet agents that offered the best behavior for what concern the reduction of platelet activation after 10 min of exposure to high shear stress were ticagrelor and cilostazol. These two agents have a common effect that is the inhibition of ADO or ADP receptors. As explained above in fact, aside from its capacity of inhibit PDE3, cilostazol is able to block the effect of adenosine. The results obtained from the present study seem to confirm the need of acting on this mechanism to better protect platelets when exposed to shear stress.

Finally, the ability of modifying the mechanical properties of platelets membrane may positively affect their response to physical stimuli. Such ability was provided by none of the antiplatelet agents tested in the present study. We believe that increasing membrane fluidity may help platelets when subjected to shear stress profiles as within VADs. So far, results suggest that the most common antiplatelet agents, which are normally used in anticoagulation management for patients treated with mechanical cardiac devices, are only partially able to protect platelets from the activation effects of physical forces encountered by flowing through cardiac assist devices. Therefore, new solutions need to be found to solve the burden of serious antithrombotic therapies, which are nowadays essential for MCS patients.

4

Dimethyl Sulfoxide: a possible antagonist of shear-induced platelet activation

This Chapter is based on:

Tran P.L.*, Valerio L.*, Yamaguchi J.T., , Brengle W., Sen N., DeCook T., Redaelli A., Slepian M.J. “Desensitization of DMSO-treated Platelets to Non-Physiological Shear Stresses and Common Agonists via Membrane Modulation.” Thrombosis Research *shared 1st author In preparation

4.1 Introduction

As emerged in the previous chapter, shear-mediated platelet activation is poorly reduced by common antiplatelet agents that can be found on the market. The fact that these agents were mainly developed to chemically inhibit platelet function may help in explaining why they are often unable to protect platelets from physical forces as the ones to which cells are subjected when flowing through MCS devices. Platelet function is mediated by several membrane proteins that are responsible for the transport of signals from the outside of the platelet to the inside organelles (Figure 4.1). The fluid dynamic conditions occurring within VADs, which are far from being physiological, may stimulate these membrane proteins, which in turn would activate platelets. This mechanism is called mechanotransduction.

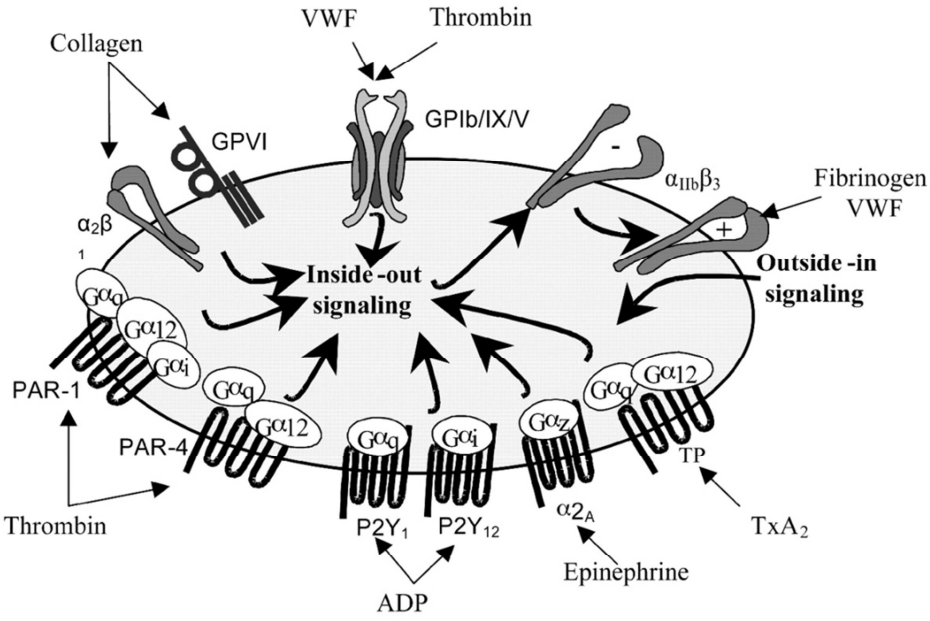

Figure 4.1. Mechanisms of mechanotransduction: the role of membrane protein in shear-mediated platelet activation (Rivera et al., 2009).

One of the mechanisms that can help to modulate the mechanotransduction effect mediated by membrane proteins is the introduction of chemical agonists which are able to affect platelet membrane fluidity, thus reducing the sensitivity of platelets to physical stimuli.

Dimethyl sulfoxide (DMSO), a small amphiphilic molecule with a hydrophilic sulfoxide group and two hydrophobic methyl groups (Figure 4.2), may be one of these agents. DMSO is an effective cryoprotectant and vasodilator (Mandumpal et al., 2011; Roth CA, 1968).

Moreover, DMSO has been used as transmembrane transporter of biochemical agents, as well as versatile solvent of several antiplatelet agents (Maddock et al., 1966; Butenas et al., 2001). For what concern the latter effect, we believe that DMSO action may actually potentiate the ability of drugs to protect platelets from shear activation.

In our study, DMSO was used to modulate intactness and fluidity of the platelet membranes with the final goal of reducing shear-mediated platelet activation. In fact, the membrane integrity and its capacity to respond to external stimuli play a key role in the mechanotransduction apparatus, which is responsible of shear-mediated platelet activation.

The effect of DMSO on the membrane hydrophobic core fluidity and its ability to induce transient water pores into the membrane were analyzed, to check whether this drug can be employed to reduce platelet reactivity to physical forces.

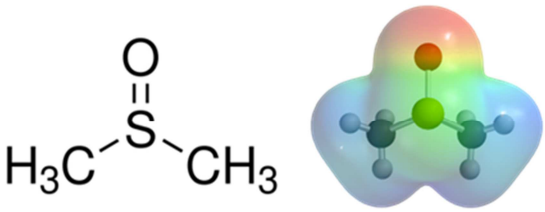

Figure 4.2. Chemical structure of DMSO. Adapted from http://commons.wikimedia.org/wiki/File:DMSO-elpot.png.

4.2 The effect of DMSO on structural and functional properties of cell membranes

Mechanotransduction refers to the mechanisms by which cells convert mechanical stimuli into chemical activity. The major membrane proteins involved in this process are integrins, which are transmembrane receptors that bring information about the chemical composition and mechanical status of the extracellular medium (ECM) into the cell. In addition to transmitting mechanical forces across membranes, they are involved in cell signaling and regulation of cell cycle, shape and motility. The chemical inhibition of this signaling process, which consists of enhancing membrane fluidity and consequently decreasing the sensitivity of these receptors to shear stress, may allow platelets to raise the threshold of their activation, thus reducing the need of antithrombotic therapies in association to MCS devices.

DMSO has been used for several pharmacological activities in cell biology, such as cryoprotection, vasodilation, membrane penetration and transport (Mandumpal et al., 2011; Roth CA, 1968; Kolb et al., 1967; Denko et al., 1967; Rammler and Zaffaroni, 1967; Elfbaum et al., 1968; Sulzberger et al., 1967; Perlman et al., 1966; Maddock et al., 1966**).**

Since in the present study we wanted to evaluated DMSO interaction with platelets, we focused our attention on its ability to 1) dissolve antiplatelet agents, 2) affect cell membranes and 3) act as antagonist to platelet aggregation. Such applications of DMSO will be described below.

In the literature DMSO was often used as a **solvent of several chemical compounds**. Indeed, DMSO has been used to dissolve antiplatelet agents during *in vitro* investigations aimed at assessing the agents effectiveness, probably potentiating their effect.

Butenas and colleagues studied platelet inhibitors in a tissue factor (TF)–initiated model of whole blood coagulation. In particular, they used low concentration of DMSO (1.6%) to dissolve dipyridamole in their investigation (Butenas et al., 2001). Furthermore, Smith used DMSO to dissolve dipyridamole and proved that prostaglandin D_2 receptors (DP) inhibitor potentiates the effect of a pyrolopyrimidine-based antifolate LY231514 (MTA) by inhibiting the transport of thymidine, essential to reverse MTA growth inhibition in leukemia and colon cancer cells (Smith et al., 2000).

Cilostazol, was found to inhibit both primary and secondary platelet aggregation induced by collagen, ADP, arachidonic acid and adrenaline, as well as to suppress the expression of p-selectin (Kariyazono et al., 2001). In their study, authors prove that the effect of cilostazol can be potentiated by the combination with forskolin dissolved in DMSO.

Forskolin is an organic compound commonly used to activate the enzyme adenylyl cyclase and increase intracellular levels of cAMP.

Moreover, Buzaid evaluated both *in vitro* and clinically the effects of adding dipyridamole to fluorodeoxyuridine (FUDR) in colorectal cancer, showing a drug-enhanced antitumor activity of FUDR. In the study, dipyridamole was dissolved in 100% DMSO and then utilized in combination with FUDR (Buzaid et al., 1989).

For what concern the **effect of DMSO on cell membranes**, Gurtovenko and colleagues performed atomic-scale molecular dynamics (MD) simulations in which they observed that DMSO exhibits three distinct modes of action, each over a different concentration range (Table 4.1). At low concentrations, DMSO induces membrane thinning and increases fluidity of the membrane hydrophobic core. At higher concentrations, DMSO induces transient water pores into the membrane. At still higher concentrations, individual lipid molecules are separated from the membrane followed by disintegration of the bilayer structure (Gurtovenko et al., 2007). These findings indicate that the choice of DMSO concentration for a given application is critical, as the concentration defines the specific mode of the solvent action. We believe that such results may be applied to platelets. In fact, an extensive knowledge of the distinct modes of action of DMSO, which in turn depends on its concentration, may promote the role of DMSO as mechanotransduction process inhibitor in shear-mediated platelet activation.

system	C_{DMSO} [mol %]	$d_{membrane}$ [nm]	area per lipid [nm^2]	DMSO mode of action
1	0.0	3.62 ± 0.01	0.690 ± 0.002	
2	2.5	3.42 ± 0.01	0.766 ± 0.002	membrane
3	5.0	3.23 ± 0.01	0.848 ± 0.002	thinning
4	7.5	3.05 ± 0.02	0.928 ± 0.003	
5	10.0	2.35 ± 0.03		membrane
6	12.5	2.13 ± 0.05		thinning
7	15.0	1.53 ± 0.06		and
8	20.0	0.52 ± 0.08		pore formation
9	25.0			
10	30.0			
11	35.0			membrane
12	40.0			disintegration
13	60.0			
14	100.0			

Table 4.1. Concentration-dependent modes of action of DMSO on phospholipid membranes (Gurtovenko et al., 2007).

The effect of DMSO on platelet aggregation was investigated by several research groups (Deutsch E, 1966; Jacob et al., 1986; Fratantoni et al., 1983). In particular, Fratantoni examined the effect of DMSO (0.1 to 10%) on platelet aggregation, release, and prostaglandin synthesis (as indicated by malondialdehyde formation) in response to thrombin, collagen, arachidonic acid and calcium ionophore. Inhibition was observed at the lowest levels of DMSO, varied with the type of stimulus, and was reversed by washing the platelets. Inhibition of aggregation, release and malondialdehyde formation were dose dependent with thrombin or collagen. DMSO did not inhibit malondialdehyde formation stimulated by arachidonic acid, nor did it consistently inhibit any function stimulated by calcium ionophore (Fratantoni et al., 1983). When platelets were stored as platelet-rich plasma at 20 to 24°C for 48 hours, with and without 5% DMSO, and subsequently washed, the platelets stored with DMSO were more reactive *in vitro*. The results obtained by the authors indicate that platelet function inhibition provided by DMSO not only was reversible, but also protected the platelets during storage. In fact, they proved as the factor limiting the use of DMSO in platelet storage is the potential toxicity provided by the solvent (Hanslick et al., 2009) in the whole circulatory system rather than on platelets.

In the light of what emerged in the studies described above about the versatility of DMSO for cell biology purposes, **the present study aims to investigate the effect of different concentration of DMSO on shear-mediated platelet activation.** In fact, a good awareness of DMSO intrinsic behavior is fundamental. Moreover, the investigation of platelets incubated with different percentages of DMSO and subjected to well described shear stress profiles may eventually reveal a new application of DMSO related to shear-mediated platelet protection.

4.3 Materials and methods

4.3.1 Experimental concept

A schematic representation of the experimental concept is reported in Figure 4.3. Details on the single steps of the procedure are explained in the following Paragraphs (from 4.3.2 to 4.3.4).

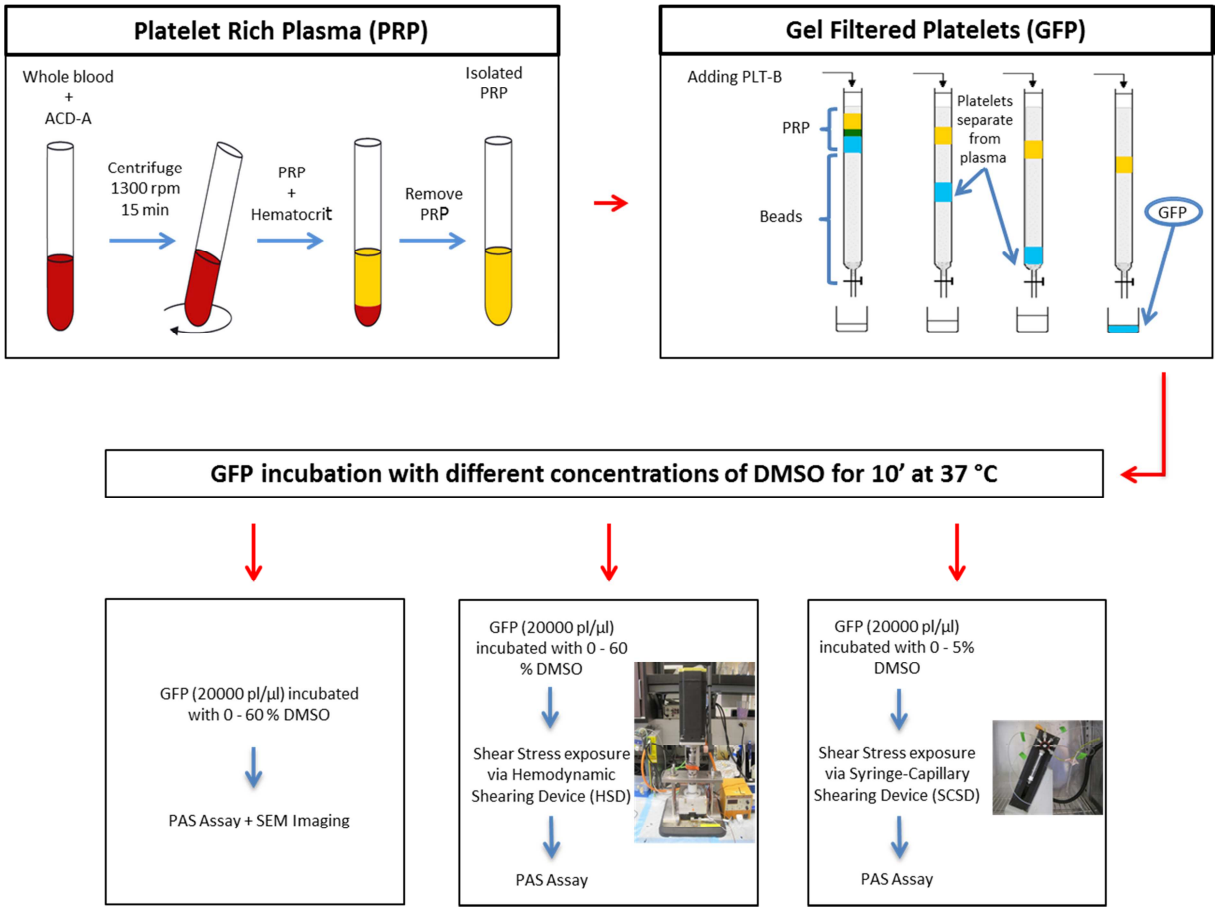

Figure 4.3. Experimental Concept. After obtaining PRP or GFP (passage through Sepharose Beads), platelets samples were either subjected to shear via HSD or SCSD and platelet reactivity was assessed using PAS assay. Adapted from http://www.glycotope.com/immunodiagnostics/clinical-diagnostics-research-reagents/thrombocytest-immune/thrombocytest-immune-handling; http://www.chemguide.co.uk/analysis/chromatography/column.html. Sephadex CL-2B.

4.3.2 Platelet samples preparation

As already mentioned in Paragraph 3.3.2, the experimental protocol utilized to obtain purified platelet refers to a procedure described in previous studies reported in the literature (Sheriff J, Bluestein D et al., 2010; Schulz-Heik et al., 2005; Jesty et al., 1999), with slight differences. Sixty healthy adult volunteers of either sex who had not taken aspirin or Ibuprofen for 2 weeks, participated to the study. Each of them signed a written informed consent, previously approved by the University of Arizona IRB. A volume of 30 ml of whole blood was drawn from each subject by antecubital venipuncture, collected in 3.8% trisodium citrate (ACD-A) (9:1,V/V) and centrifuged at 1300 rpm for 15 min. With this procedure, platelet-rich plasma (PRP) was separated from red blood cells. At this point, PRP was used for producing gel-filtered platelets (GFP) by filtration through a 175-ml column of Sepharose 2B beads (60-200 μm diameter, 2% agarose; Sigma-Aldrich, St. Louis, MO) equilibrated in platelet buffer (Neuenschwander et al., 1988). The resulting GFP were counted using Z2 Particle Counter (Beckman Coulter, Miami, FL) and diluted with platelet buffer to a concentration of 20,000 pl/μl. At this point of the procedure, the testing solutions were incubated with or without DMSO at different concentrations for 10 min at 37°C and samples were then ready for experimental campaigns.

4.3.3 Experimental Campaign

The experimental campaign performed in this study consisted in three experiments. In the static condition experiment, platelets were incubated with different percentages of DMSO and platelet activity state detected.

In the hemodynamic shearing device (HSD) dynamic experiments, platelets incubated with DMSO were subjected to both constant and dynamic shear stress profiles using the HSD.

In the syringe-capillary shearing device (SCSD) dynamic experiments, to assess the ability of DMSO to protect platelets after exposure to shear stress peaks not reachable via HSD, samples were exposed to hyper shear using the SCSD.

After each experiment, platelet activity state was measured using the platelet activity state (PAS) assay (Jesty et al., 1999), a modified prothrombinase method that was described in Paragraph 3.3.5. A detailed description of each type of experiment is reported hereinafter.

4.3.3.1 Effect of DMSO on platelet activation under static condition

The reactivity of platelets to different concentrations of DMSO was first tested under static conditions. 4 ml of GFP solution (20000 pl/µl) were incubated for 10 min at 37 °C with 0%, 0.95%, 2,5%, 5%, 10%, 15%, 20%, 25%, 40% and 60% of DMSO, where percentages are referred to total volume of sample solution. PAS assay was performed at each concentration of DMSO tested. Platelets incubated with DMSO were also fixed and prepared for SEM imaging, as described in Paragraph 4.3.4.

4.3.3.2 Effect of DMSO on platelet activation after exposure to shear stress via HSD

A set of experiments was performed to find out the level of sonication to be used for normalization of PAS data resulting from the DMSO dynamic experiment. As in the static experiment, 4 ml of platelets (20000 pl/µl) were incubated for 10 min at 37 °C with 0%, 0.95%, 2,5%, 5%, 10%, 15%, 20%, 25%, 40% and 60% of DMSO. Then, they were subjected to different sonication levels using a Branson Sonifier 150 with microprobe (Branson, MO) for 10 s. The tested conditions were 0, 20, 25, 30, 35, 40, 45 and 50% of the maximum output power, that is 150 W. The 0% sonication condition corresponded to the static condition explained in Paragraph 4.3.3.1.

After definition of the correct level of sonication to use for the normalization of the data obtained from PAS assay, new experiments were conducted to investigate the effect of DMSO after platelets exposure to different shear stress profiles in the HSD. The results obtained in each experiment were compared to the reference value of platelets subjected to shear and incubated without DMSO.

Before each experiment, GFP solutions (20000 pl/µl equilibrated using platelet buffer) were incubated with different aliquots of DMSO for 10 min at 37 °C. The percentages in volume of DMSO were 0%, 0.95%, 2.5%, 5%, 10%, 15%, 20%, 25%, with the exception of particular conditions for which we investigated the effect of 40% and 60% DMSO as well. After incubation with DMSO, samples were subjected to the shear stress conditions via HSD. Details regarding the HSD are reported in literature (Girdhar et al., 2008), as well as in Chapter 2. A graphical explanation of the shearing profiles to which platelets were subjected through the device is represented in Figure 4.4.

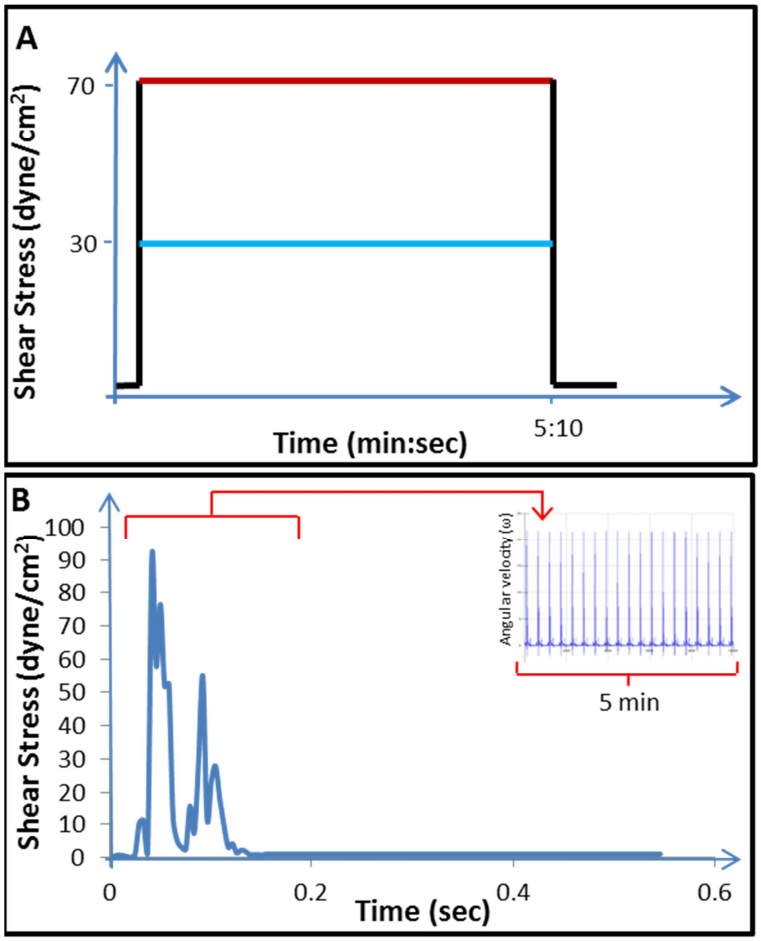

Figure 4.4. Constant and dynamic shear stress waveforms. Gel-filtered platelets were exposed to (A) variable magnitude of constant shear stress and (B) dynamic waveform with variable angular velocities representing a single cycle of generic Debakey VAD (50th percentile of PDF). Time exposure for both shear conditions were equalized to a total experimental duration of 5 min. The dynamic shear stress profile (B) visualized in Figure correspond to one cycle. To match the exposure time of constant waveform, the dynamic profile was repeated 550 times within the HSD.

The dynamic shear stress profiles extracted from the DeBakey VAD and used in the study were implemented thanks to the application of the DTE (Device Thrombogenicity Emulator), developed by research groups in Stony Brook (New York) and University of Arizona (Tucson) already described in literature (Girdhar et al., 2012). DTE allows to model flow path within VADs using CFD simulations, thus tracking down and capturing the loading history of platelets in the flow field along trajectories that may drive them beyond the activation threshold. These cumulative stresses are then collapsed into probability density functions (PDFs) representing the device 'thrombogenic footprint'. The extraction of particular profiles from PDFs waveforms and the implementation of such shear stresses via HSD allowed to assess the effect of DMSO in protecting platelets under conditions that are similar to those encountered in MCS devices.

In the present study, the effect of DMSO on platelets was investigated by subjecting GFP pre-treated with the agonist to different shear stress profiles, either constant or dynamic,

extracted from the PDF of a Debakey VAD. Anyhow, the limitations of HSD did not allow to replicate range of shear stress peaks of 10^3 dyne/cm^2, as CFD simulations within VADs underlined. Thus, a factor scale of 15 was used as trade off to reduce the shear stress to levels feasible for the machine but still interesting for the purpose of the study, that was to investigate the effect of DMSO under shear profiles similar to the ones encountered *in vivo* by platelets. Despite this limitation, the HSD mimicked the extremely rapid dynamic loading waveforms that platelets experience along trajectories while passing through VADs, allowing to investigate platelets behavior under shear conditions never investigated before in literature.

For what concerns the constant conditions, platelets were subjected via HSD to 30 and 70 dyne/cm^2 for a total time of 5 min. On the other hand, in the dynamic condition, the loading pattern implemented via HSD was extracted from the PDF of a DeBakey VAD and corresponded to the stress accumulation level found at the 50th percentile of the total PDF waveform (Dynamic_50, further details were exposed in Chapter 3). Since the actual exposure time characterizing such extracted profile was few ms, the loading waveform was repeated cyclically for a total of 550 loops within HSD to match the total duration of the experiments under constant shear stress conditions (5 min).

4.3.3.3 Effect of DMSO on platelet activation after exposure to shear stress via SCSD

To overcome the limitations of the HSD in terms of maximum shear stress peak, we studied DMSO-treated platelets also through the SCSD, although this device was less able to subject platelets to complex dynamic shear stress profiles. SCSD, built in collaboration between Stony Brook University (Prof. D. Bluestein) and University of Arizona (Prof. M. J. Slepian) is a syringe-capillary pump system. Within the device, platelets are exposed to different shear stresses by cyclically passing through capillaries with defined geometries at a controlled flow rate. In the SCSD, platelets were exposed to the experimental shear stress while passing through capillaries positioned downstream of a computer controlled syringe pump (outflow position). In order to refill the pump and allow a cyclic exposure of platelets to the experimental conditions, an inflow conduit was also connected to the system. Further details about this device was provided in Chapter 2, as well as in (Sheriff J, 2010). All parameters of the experiments with SCSD corresponding to the outflow configurations are reported in Table 4.2. The inflow conduit was the same for all shear stress investigated, and consisted in a silicon tube with inner diameter ϕ = 2mm and length L = 200 mm. Flow rate within the tube

was 71 ml/min, thus subjecting platelets to an actual shear stress of 15 dyne/cm^2 for an exposure time of 0.53 s each cycle.

Shear Stress	**Capillary**		**Flow Rate**	**Single pass $t_{exposure}$**	**N Loops**	**Total $t_{exposure}$**	**Duration Experiment**
[dyne/cm^2]	**Φ** [mm]	**L** [mm]	[ml/min]	[s]		[s]	[s]
140	0.8	30	43	0.212	12	2.544	108
400	0.8	30	122	0.074	37	2.738	198
680	0.8	30	203	0.044	74	3.256	339
880	0.8	40	265	0.045	74	3.33	319
1440	0.38	30	46	0.044	74	3.256	639
880	0.8	40	265	0.045	477	21.5	2056

Table 4.2. Parameters used for the SCSD experiment. The duration of each experiment was calculated as (N Loops)(t cycle), where t cycle was the duration of a single cycle within the device, that was different among shear stress conditions and took into account both inflow and outflow passages.

The effect of DMSO was assessed at high shear stress stimulations, corresponding to 140, 400, 680, 880 and 1440 dyne/cm^2. The choice of such high shear stresses was to match peaks that platelets experience through VADs, which lie in the order of 10^3 dyne/cm^2. The volume of sample used for each experiment was 4 ml. In one cycle within the device platelets were exposed to the actual shear stress for variable time intervals between 0.044 to 0.212 s. To allow platelets to experience shear stress for prolonged period of time, we thus looped the experiments within the SCSD. For example, the total exposure time to the actual hyper shear experienced by platelets at 880 dyne/cm^2 was 3.33 s, corresponding to 74 loops within the device (Figure 4.5).

In the SCSD experiments, to preserve platelets integrity while extending the results obtained during the HSD campaign, only platelets incubated with 0%, 0.95%, 2.5% and 5% of DMSO were studied.

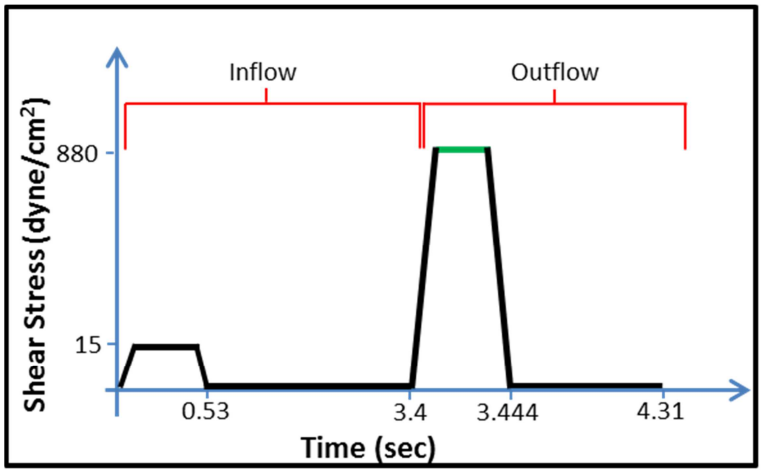

Figure 4.5. Shear stress profiles in the SCSD. Example with a shear stress peak equal to 880 dyne/cm^2. Geometrical characteristics of the inflow and outflow tubes didn't allow to subject all platelets to the same shear stress for all duration of the two phases. The duration of a single loop (t cycle) was 4.31 s.

We also decided to increase the shear stress exposure with the SCSD at 880 dyne/cm^2, expanding the exposure time in the device to 21.5 s, i.e. 477 loops (Table 4.2 – last row). In this configuration, the Total Stress Accumulation (TSA) to which platelets were exposed in the SCSD was of the same order of magnitude of the one experienced after 5 min of exposure at 30 dyne/cm^2 in the HSD. Because the time necessary to refill the syringe pump for each cycle was 3.4 s, the whole experiment lasted 34 min. PAS assay was again performed to assess the thrombin release level after exposure to the investigated shear profiles.

Finally, for a selected condition (Table 4.2 – last row) we checked the viability of platelets after treatment with DMSO and hyper shear stress exposure. This assessment was performed by further incubating samples pre-treated to hyper-shear (with and without DMSO (5%, V/V)) with arachidonic acid (AA) (0.5 mM) for 1 min. AA was meant to elicit the activation of viable platelets; activation was assessed with PAS assay.

4.3.4 Preparation for SEM imaging of platelets treated with different percentages of DMSO

During the experimental campaign, SEM images of platelets in several conditions of interest were acquired. A detailed visual inspection of DMSO-treated platelets was mandatory to better investigate the effect of DMSO. The procedure for fixing platelets and performing SEM imaging will be described hereinafter.

After DMSO exposure, 150 µl of living platelets (20,000 pl/µl) were added to glass coverslips and incubated for 15 min. After removal of the excess sample, a thin film atop the slide was left by using a transfer pipet, to which 300 µL of 100% Fixator solution (2% V/V of Glutaraldehyde in platelet buffer) was added. The solution obtained in this way was incubated under hood for 30 min and the subsequent washing phase started. In particular, samples were first washed with distilled water, later with 100% Ethanol and finally with 100% Hexamethyldisilazane (HMDS). Each different wash was performed 5 min apart from the others. After the just mentioned procedure, samples were left to air-dry overnight, mounted on double-sided carbon tape and brought to the imaging facilities for SEM.

4.3.5 Statistical analysis

Statistical tests were performed using SigmaPlot (Systat Software Inc). Shapiro Normality test was runned for all population of data before significance tests. Data corresponding to platelets incubated with different percentages of DMSO were analyzed after exposure to shear stress profiles via HSD or SCSD and compared with control (0% DMSO). Either parametric or non-parametric (Kruskal-Wallis) one-way ANOVA were performed depending on the distribution of the data analyzed. Single comparison t-test was used in two-population comparisons when possible, while Mann-Whitney Rank Sum Tests were performed when non-parametric analysis between two groups were needed. Differences were considered significant if $p < 0.05$.

4.4 Results

4.4.1 The effect of DMSO on platelet activity under static condition

The results of the static condition experiment are shown in Figure 4.6, where the thrombin generation curve is plotted. This curve was obtained with the spectrophotometer in a total reading period of 7 min that followed the phase of incubation with different concentrations of DMSO (Figure 4.6). The reactivity of platelets to different DMSO percentages can be assessed by looking at the change in sample absorbance per unit time. The number of experiments performed was 6, with blood samples from at least 3 donors.

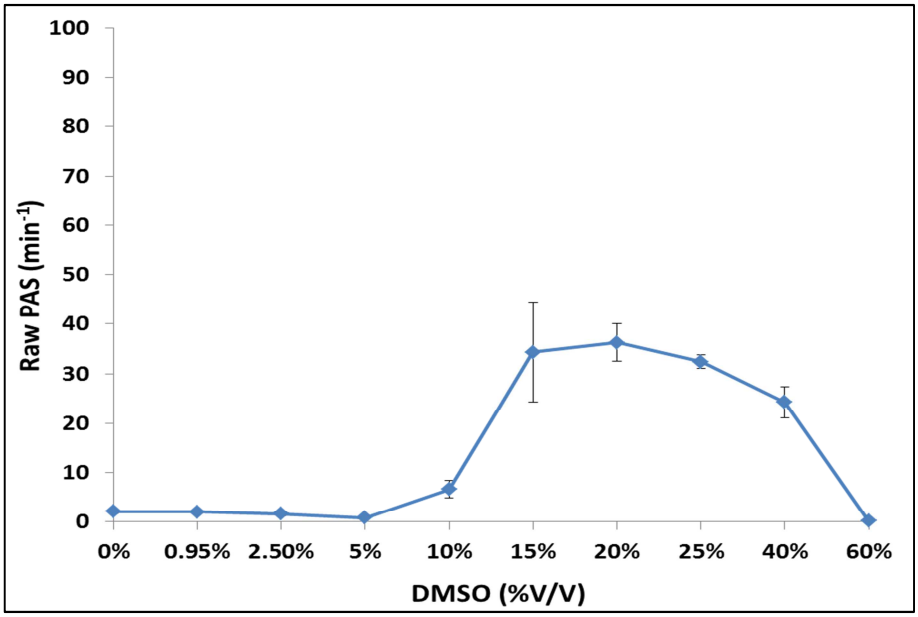

Figure 4.6. The effect of DMSO on the activity of platelets under static condition. Human platelet activity state when exposed to varying DMSO concentration ((%V/V), 10 min exposure). N Expt = 6 from at least 3 different donors. Data are represented as mean ± SEM.

As emerged from the results obtained, platelets showed no activation when incubated with a total volume of DMSO ≤ 5%. For 10 ≤ % DMSO ≤ 40, platelets resulted activated, with a peak of thrombin release at a concentration of DMSO equal to 20%. This phenomenon may be due to a platelet sensitization provided by DMSO at those concentrations, thus resulting in an increased level of activation detected with the PAS assay. Platelet solutions with content of DMSO ≥ 40% seemingly showed a diminishing platelet activation trend. It should be noted that activation is calculated as thrombin release, whereas the toxicity of DMSO at high concentrations may cause platelet membrane lysis, thus affecting platelet viability or responsiveness at least. Such effect is clearly visible in the SEM images in Figure 4.7.

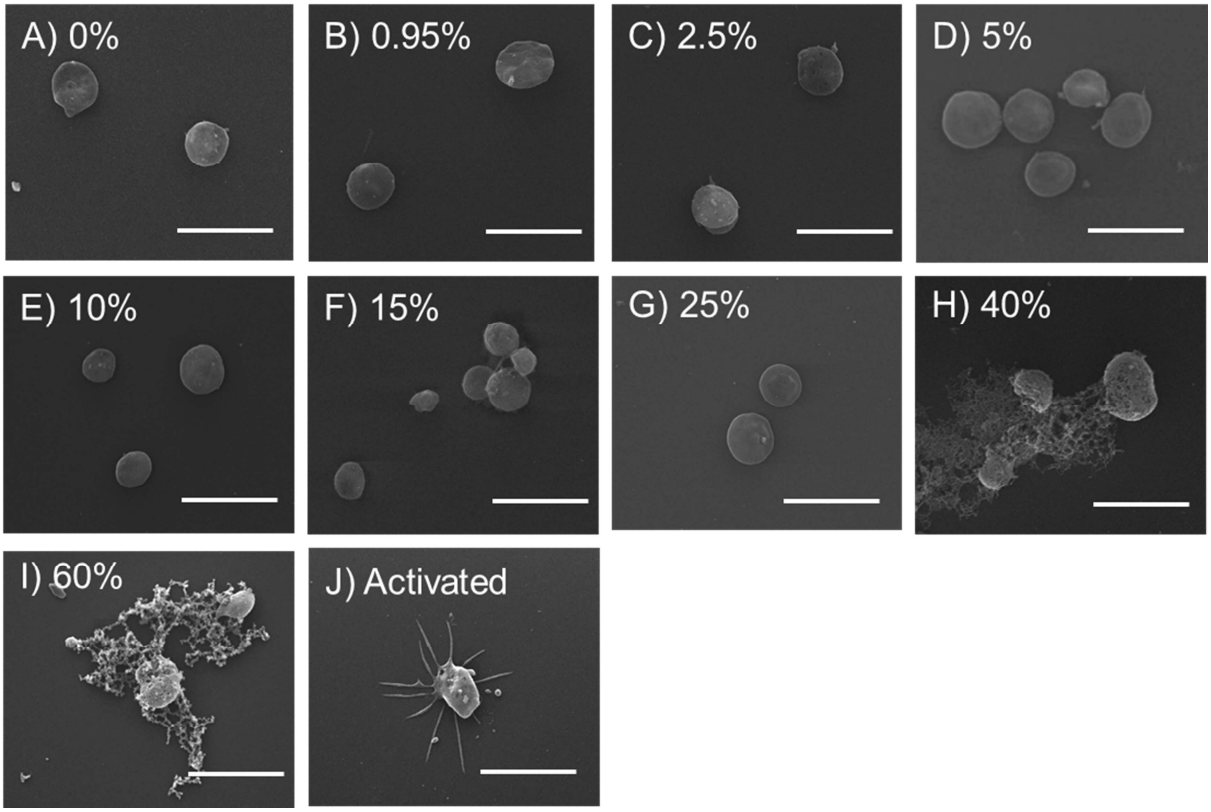

Figure 4.7. SEM of platelets at 20000x of magnification incubated with different concentration of DMSO for 10 min (A - I). In J is represented an SEM image of a fully activated platelet.

In fact, for concentration of DMSO < 40%, platelet membrane appeared intact, with no changes in shape. At higher concentration of DMSO instead, as visible in Figure 4.7-H,I, a damage of the cell occurred, with disruption of platelet membrane and release of all granules or other platelets content on the carbon tape. In figure 4.7-J is represented a platelet activated via sonication, where changes in shape and presences of micro granules and pseudopods are clearly evident.

4.4.2 The effect of DMSO on platelet activity under different level of shear performed via HSD

Preliminarily, platelets incubated with DMSO were exposed to different level of sonication and the thrombin generation curves relative to this experiment are shown in Figure 4.8.

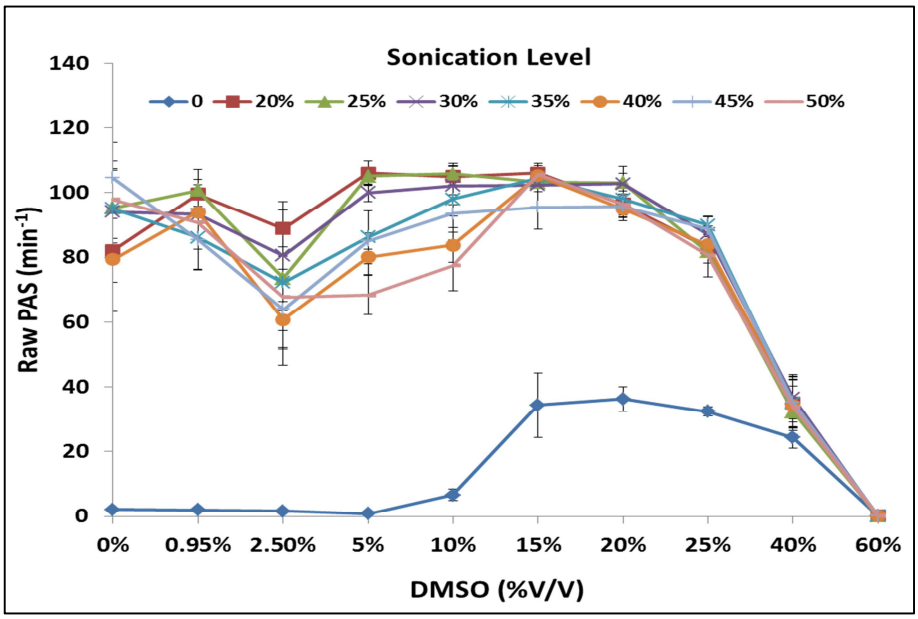

Figure 4.8. The effect of DMSO on the activity of platelets exposed to different % of sonication. Human platelet activity state after exposure to varying DMSO concentration ((%V/V), 10 min exposure) and then sonicated at different level (0 – 50%, i. e. 0 – 75 W, 10 s exposure). N Expt= 6 from at least 3 different donors. Data are represented as mean ± SEM.

The data obtained displayed a tendency to the reduction in thrombin generation for platelets treated with 2.5% of DMSO (difference not statistically significant), with a subsequent raise to a level of activation similar to the starting point values. The final drop in samples treated with DMSO > 25% has to be intended as the achievement of a level of toxicity provided by DMSO harmful for cells, as already evidenced for non-sonicated samples. Despite data obtained showed non statistically significant differences for all DMSO concentration tested ($p > 0.2$), 20% of the maximum power supplied by the sonicator, i.e. 30 W, appeared to maintain the level of platelet activation almost constant for the range of DMSO of interest for the study (0 – 25%). We thus decided to normalize platelets activation obtained during the experiments with shear stress exposure using samples activated via sonicator at 30 W for 10 s.

Platelets incubated with different DMSO concentrations were then subjected to shear stress profiles via HSD. The results obtained at 30 dyne/cm^2, 70 dyne/cm^2 and after exposure to a shear profile extracted from the PDF of the Debakey VAD corresponding to the 50th percentile of the total waveform, scaled for the limitation of the device, are exposed

hereinafter. After 5 min in the HSD, samples (500 µl) were taken using a 1 ml syringe appropriately connected to the shearing device. The PAS values detected were normalized with respect to platelets fully activated via sonicator at 30 W. The platelet activation trend obtained at 30 dyne/cm^2 is shown in Figure 4.9.

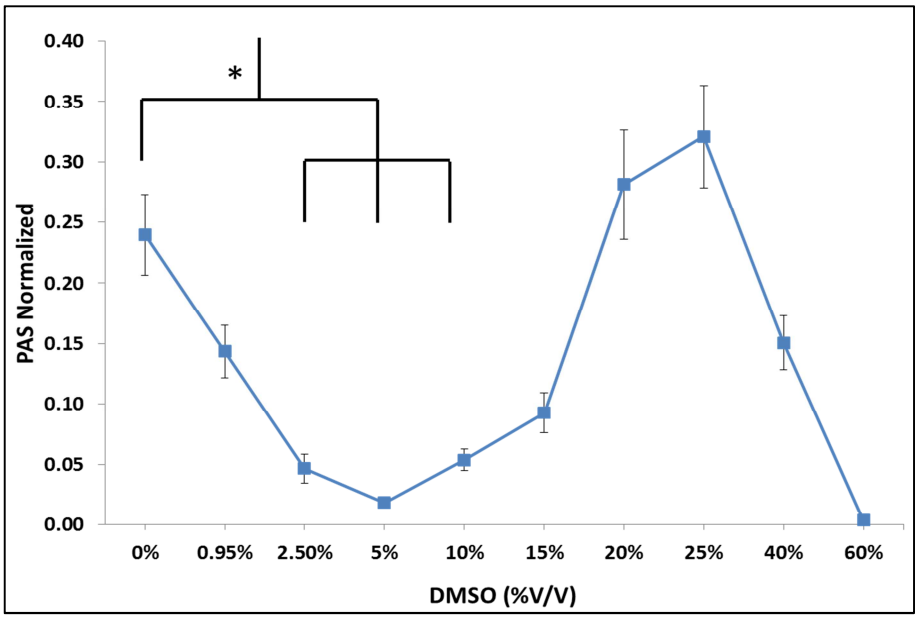

Figure 4.9. Ability of DMSO to modulate shear-mediated human platelet activation. Pre-treatment with DMSO at specified concentrations (10 min., 37^{O}C). Shear exposure via HSD (30 dyne/cm^2, 5 min., 37^{O}C). N Expt = 8+ from at least 3 different donors. Data are represented as mean ± SEM. * $p < 0.05$

The range of concentrations of DMSO tested in this configuration was 0% - 60%. Data showed as DMSO already provided a reduction in platelet activation at very low concentration (0.95%) with major effects between 2.5% and 10% ($p < 0.05$). At higher concentrations of DMSO (15% - 25%) platelets were instead activated at levels corresponding to samples non treated with DMSO. Moreover, concentrations of DMSO > 40% showed a toxic behavior, with a reduction in thrombin release monitored by the PAS assay that was attributed to the lysis of platelets membrane instead of protection after shear stress exposure.

A similar pattern was found at 70 dyne/cm^2, as reported in Figure 4.10.

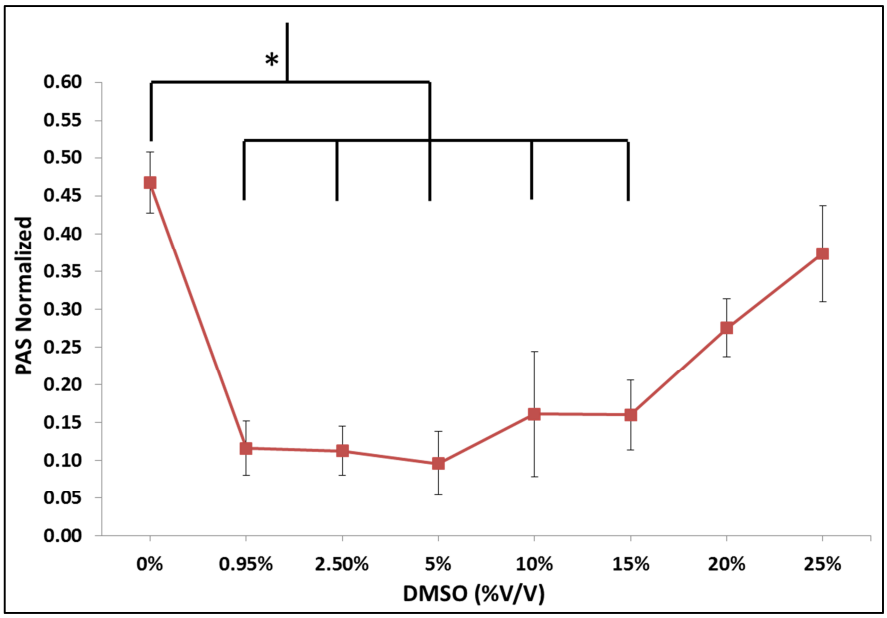

Figure 4.10. Ability of DMSO to modulate shear-mediated human platelet activation. Pre-treatment with DMSO at specified concentrations (10 min., 37^{O}C). Shear exposure via HSD (70 dyne/cm^2, 5 min., 37^{O}C). N Expt = 8+ from at least 3 different donors. Data are represented as mean ± SEM. * $p < 0.05$

Based on results of both the experiment in static condition (Paragraph 4.4.1) and after exposure to 30 dyne/cm^2, the effect of DMSO on platelet subjected to higher shear stress conditions via HSD was investigated in the range of 0% - 25%. The results obtained showed a similar behavior compared to 30 dyne/cm^2. Low concentrations of DMSO resulted able to protect platelet from shear-mediated platelet activation, with a statistical significance at concentrations < 15% ($p < 0.05$).

Results obtained after exposure of platelets to the dynamic shear profile extracted from the DeBakey VAD are described in Figure 4.11.

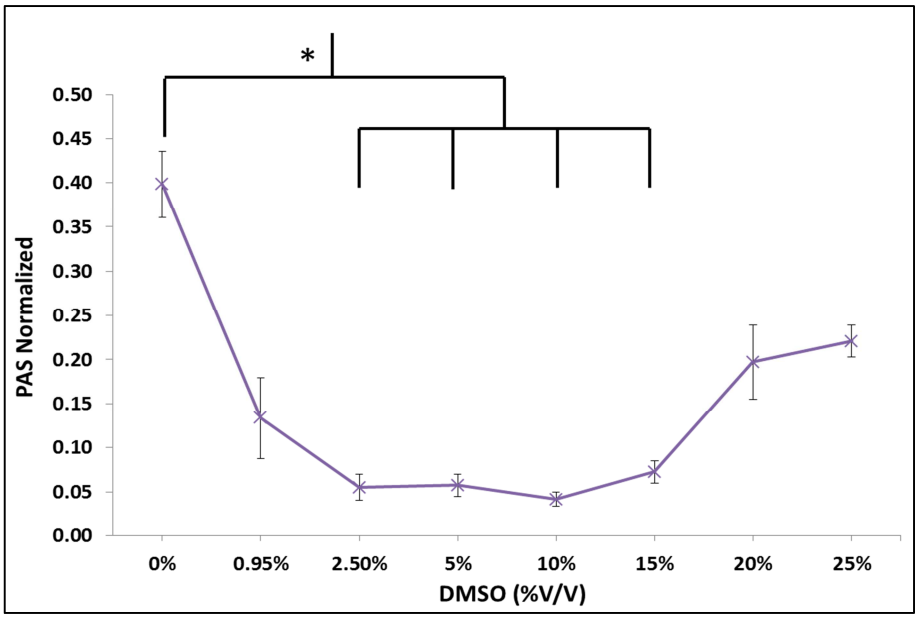

Figure 4.11. Ability of DMSO to modulate shear-mediated human platelet activation. Pre-treatment with DMSO at specified concentrations (10 min., 37^{O}C). Shear exposure via HSD (50th percentile of PDF from Debakey VAD, 5 min., 37^{O}C). N Expt = 8+ from at least 3 different donors. Data are represented as mean ± SEM. * $p < 0.05$

DMSO showed a protection effect even after exposing platelets to this shear stress profile, confirming its powerful contribution in shear-mediated platelet activation inhibition at low concentrations (DMSO < 15%, $p < 0.05$ compared to control). Finally, Figure 4.12 shows the comparison of the platelet activation patterns obtained subjecting platelet samples incubated with 0 – 25% DMSO to the three different shear profiles via HSD.

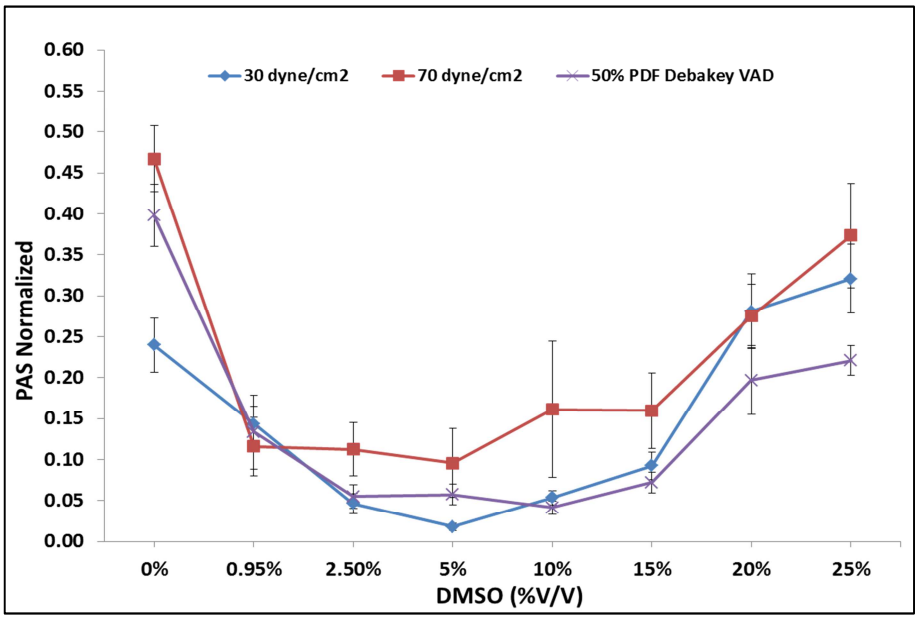

Figure 4.12. Ability of DMSO to modulate shear-mediated human platelet activation. DMSO pre-treatment at specified concentrations (10 min., 37^{O}C). Comparison between shear stress profiles (5 min., 37^{O}C). Data are represented as mean ± SEM.

4.4.3 The effect of DMSO on platelet activity after exposure to hyper shear

The effect of DMSO on shear-mediated platelet activation was investigated with the SCSD. The shear stresses to which platelets were exposed via SCSD were 140, 400, 680, 880 and 1440 dyne/cm^2, for a total exposure time that varied depending on the shear stress. Both exposure time and stress accumulation (SA) tested in the present study via HSD and SCSD were summarized in Table 4.3 A-B.

Shear Stress Conditions (τ_w) [dyne/cm^2]	**Exposure Time ($t_{exposure}$)** [s]	**Total Stress Accumulation (TSA)** [dyne s/cm^2]
30 dyne/cm^2	120	3600
	300	**9000**
	600	18000
70 dyne/cm^2	120	8400
	300	**21000**
	600	42000
50th percentile of PDF Debakey VAD	120 (220 cycles)	96
	300 (550 cycles)	**239**
	600 (1100 cycles)	478

Table 4.3-A. Description of shear stress conditions tested via HSD.

The TSA for the constant shear stress performed via HSD was calculated through the following equation:

$$TSA = \tau\, t_{exposure}$$

Where τ is the shear stress (30 or 70 dyne/cm^2) and $t_{exposure}$ is the exposure time, calculated in seconds.

For what concern the dynamic shear stress waveform extracted from the PDF of the DeBakey VAD, the Stress Accumulation (SA) obtained for each cycle within the HSD was determined using:

$$SA_{1\,cycle} = \sum_i (t_{i+1} - t_i)\left(\frac{\tau_{i+1} + \tau_i}{2}\right)$$

Where i indicate the time step in which the waveform was divided and the shear stress calculated at each time point. According to the latter equation, TSA for the dynamic

waveform was thus calculated by multiplying the $SA_{1\ cycle}$ to the number of cycles performed in the HSD.

Shear Stress Condition (τ_w) [dyne/cm^2]	**Average Shear Stress (τ_{avg})** [dyne/cm^2]	**Exposure Time ($t_{exposure}$)** [s]	**Total Stress Accumulation (TSA)** [dyne s/cm^2]
140	70	2.544	226
400	200	2.738	695
680	340	3.256	1414
880	440	3.33	1776
1440	720	3.256	2660

Table 4.3-B. Description of shear stress conditions tested via SCSD. All parameters correspond to the outflow configuration. Total Stress Accumulation (TSA) is calculated considering also the shear stress due to the refilling of the syringe (Inflow exposure) for each cycle of exposure to actual shear stresses tested.

The TSA obtained during the experiments with SCSD was calculated as:

$$TSA = \left(\tau_{avg-in}\ texp_{in} + \tau_{avg-out}\ texp_{out}\right) N_{loops}$$

Where $\tau_{avg\ -\ in}$ and $\tau_{avg\ -\ out}$ are the average shear stress calculated in the inflow or outflow capillaries while $texp_{in}$ and $texp_{out}$ are the exposure time during a single passage of platelets in the inflow and outflow tubes.

Assuming that the shear stress decreases linearly from a maximum at the wall (τ_w) to 0 at the center (Sheriff J, 2010), the average shear stress was approximately calculated as:

$$\tau_{avg} = 0.5\tau_w$$

It appears clear that the TSA experienced via HSD was consistently more elevated compared to the one tested with SCSD.

The experiments performed with the SCSD were different compared to the ones realized using the HSD. The investigation of the effect of DMSO under hyper shear conditions, not achievable via HSD, provided a fundamental further information for the characterization of DMSO as platelet activation inhibitor. During this set of experiments we studied the effect of DMSO on platelets after exposing samples incubated with concentrations of DMSO of 0.95%,

2.5% and 5% to hyper shear. The results obtained with the HSD established how this particular range of DMSO concentrations was always able to protect platelet from shear mediated platelet activation, thus indicating that those concentrations were of the most interest for further investigations.

Results obtained with the SCSD are represented in Figure 4.13.

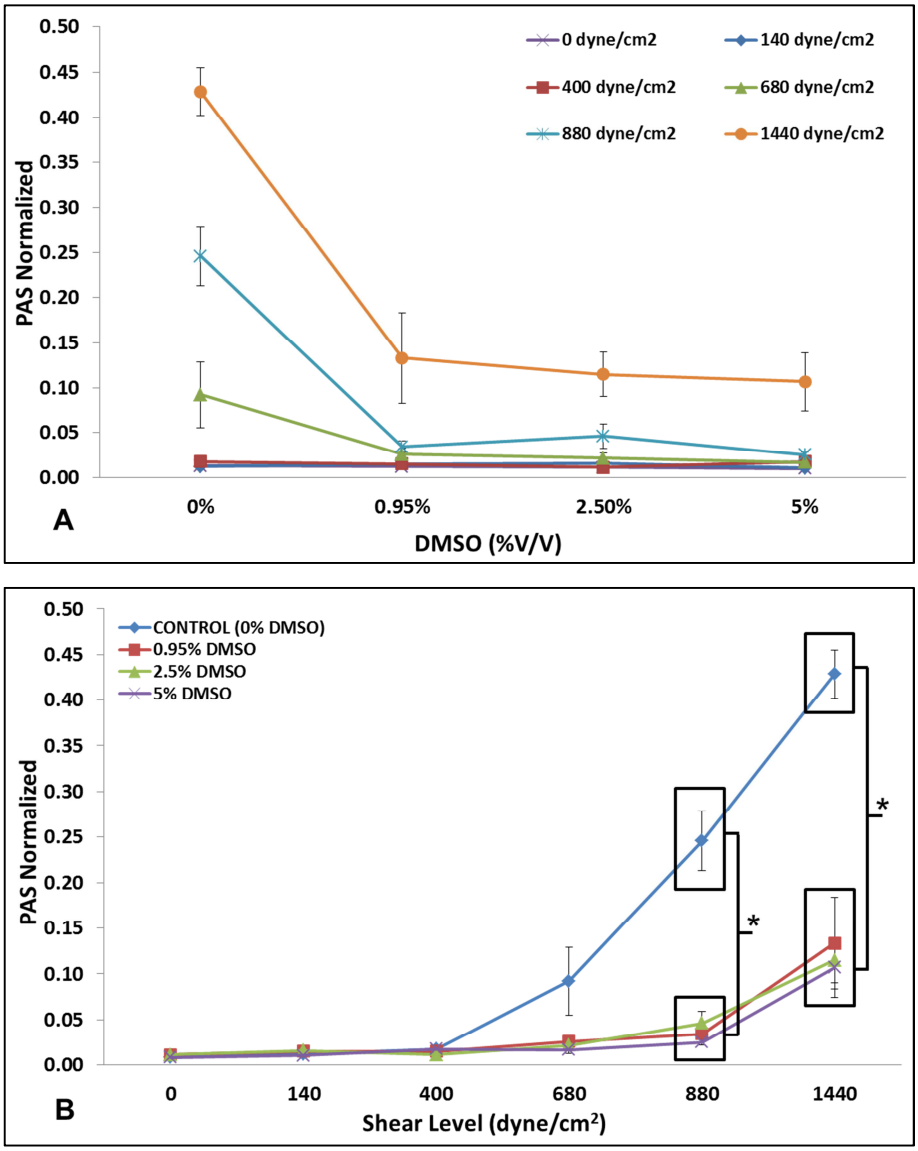

Figure 4.13. Ability of DMSO to protect platelets exposed to hyper shear conditions. Pre-treatment with DMSO at specified concentrations (10 min., 37^{O}C). Shear exposure via SCSD (140,400,680,880,1440 dyne/cm^2 37^{O}C). N Expt = 6+ from at least 3 different donors. Data are represented as mean ± SEM. * $p < 0.05$

As displayed in figure 4.13-B, platelets pre-treated with DMSO showed a reduced activation after exposure to shear stress, with significant differences compared to control after exposure to 880 and 1440 dyne/cm^2.

A further experiment was conducted in an attempt to compare the data obtained with HSD and the SCSD. Indeed, the SCSD, although able to achieve higher shear stress peaks during experiments, did not provide platelets with the same SA experienced during the experimental campaign with HSD. In order to reach a TSA that was similar to what experienced by platelets after 5 min of exposure at 30 dyne/cm^2 via HSD, we subjected platelets to a shear stress level of 880 dyne/cm^2 via SCSD for a total exposure time of 21.5 s. The calculated SA obtainable in this condition was actually higher compared to the one reached via HSD, but of the same order of magnitude (11446 vs 9000 dyne s /cm^2). This experiment allowed to test platelets at different shear stress peaks but similar SA. The results of the comparison between samples subjected to shear in the two configurations are represented in Figure 4.14.

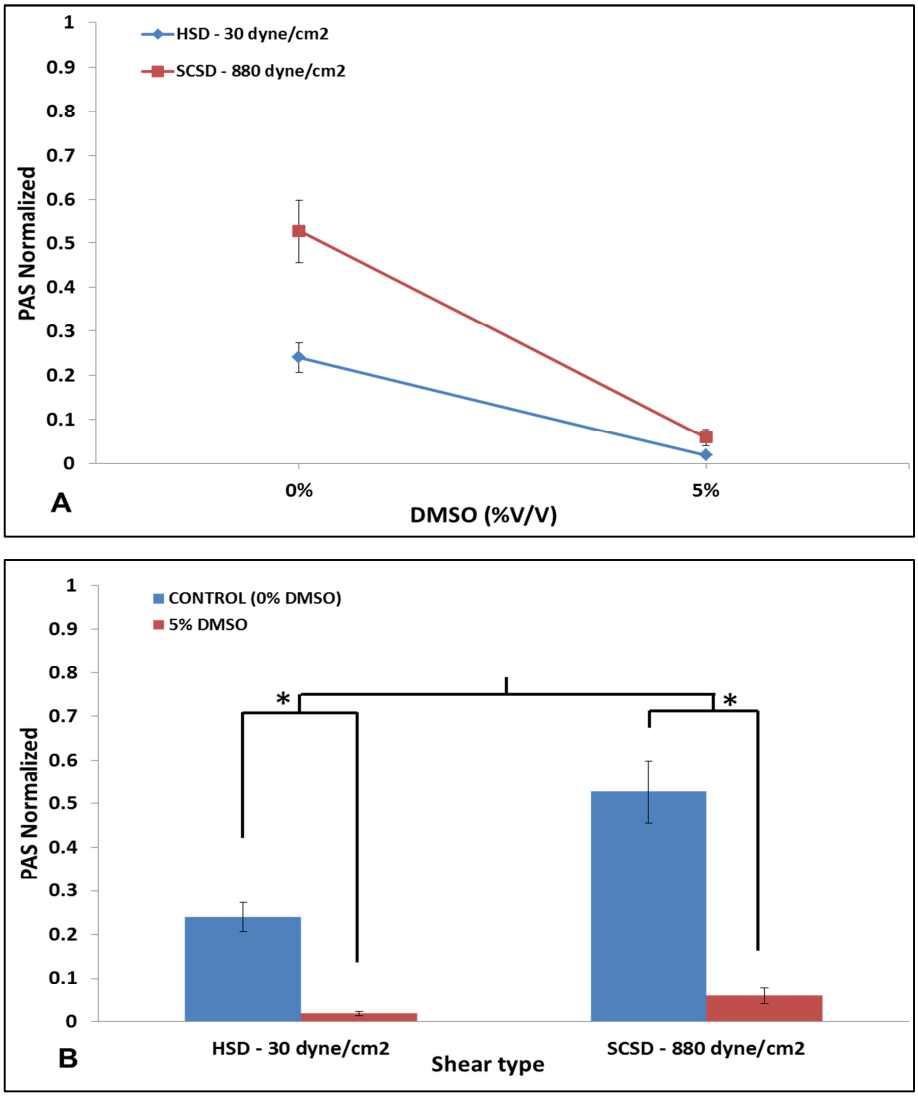

Figure 4.14. Characterization of platelet activation after exposure to similar TSA via HSD and SCSD – N Expt = 6+ from at least 3 different donors. Data are represented as mean ± SEM. * $p < 0.05$

The behavior of platelets exposed to shear stress in the two different devices seemed different. In fact, although the SA was comparable in the two configuration tested, control samples obtained with SCSD displayed a significantly higher platelet activity state compared to control samples obtained via HSD (0.53 vs 0.24, $p < 0.05$). The level of PAS detected after platelets exposure of 30 dyne/cm^2 via HSD for 5 min was comparable to the one obtained at 880 dyne/cm^2 via SCSD in the previous configuration (Figure 4.13), where the exposure time was only 3.33 s (0.24 vs 0.25). Anyway, platelets pre-treated with 5% DMSO showed a consistent reduction in platelet activation for both experiments compared to their respective controls ($p < 0.05$), confirming the protective effect offered by the reagent investigated.

In order to verify the viability of platelets after exposure to hyper shear stress conditions, we perform an assessment in which we further incubate samples pre-treated to hyper-shear for 1 minute with arachidonic acid (AA). AA was meant to elicit the activation of viable platelets. Platelets incubated with 5% DMSO were subjected to 880 dyne/cm^2 via SCSD and then further incubated with 0.5 mM of AA before PAS assay. We investigated the effect of 5% DMSO in solution because this was the concentration that provided the largest reduction during the previous experiments. For each test a control sample without DMSO was run and the results obtained are shown in Figure 4.15.

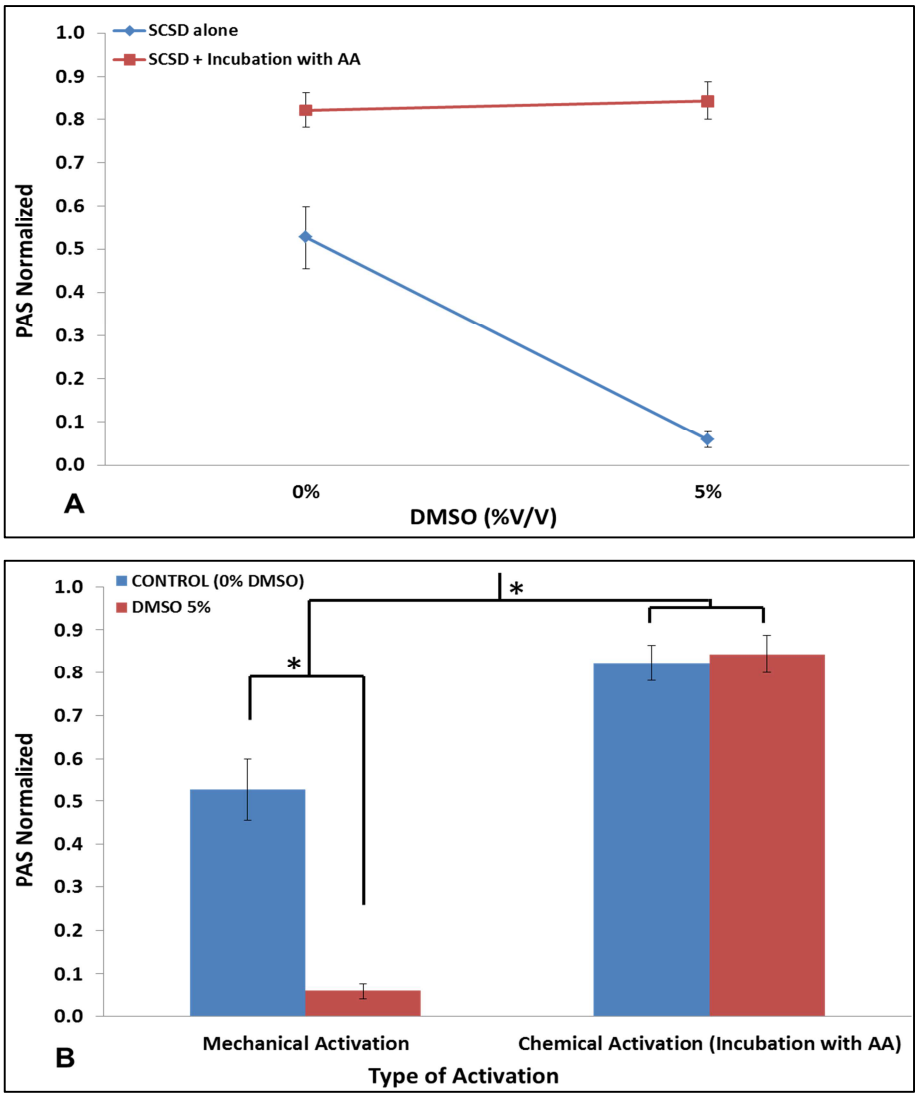

Figure 4.15. Different platelets response to mechanical and chemical activation at 800 dyne/cm^2 in the SCSD (TSA = 11446 dyne s/cm^2). N Expt = 6+ from at least 3 different donors. Data are represented as mean ± SEM. * $p < 0.05$

There was a significant difference between the effect of DMSO with respect to mechanical or mechano-chemical stimuli. Platelets treated with 5% DMSO and subjected only to a mechanical stimulus presented a reduced activation compared to the control group, while no differences between the two groups were detected in samples extracted from the same experiment and afterwards mixed with AA. PAS data certified a platelets reactivity to the chemical agonist after shear exposure, thus confirming the viability of cells.

Moreover, it was proved that DMSO provides a good protection against platelets activation driven by physical forces, but it has very little effect when the activation is mediated by a mechano-chemical process.

This method is not the only one that can be used to assess platelets viability. In fact, SEM images of platelets in the different experimental conditions will provide extremely

important information about platelets morphology and shape changes, allowing to check whether DMSO in combination with shear stress offered an actual visible modification of platelets structure.

4.5 Discussion

In the present study we assessed the ability of DMSO to protect platelets from shear stress exposure, a potentially beneficial effect that we relate to the capability of DMSO to modify platelet membrane structural characteristics.

DMSO is a powerful chemical compound, used in cell biology for several purposes including platelet aggregation inhibition and cell membrane modification. Moreover, studies regarding antiplatelet antagonists mainly used DMSO as a vehicle to help drugs penetration into the cellular membrane, or as a solvent (Peerschke et al., 2004; Kariyazono et al., 2001).

To the best of our knowledge, there is only one study, performed by Asmis and colleagues, regarding the ability of DMSO to impair thrombus formation (Asmis et al., 2010).

Based on its chemical properties, we decided to investigate the effect of DMSO on shear-mediated platelet activation. Low percentages of DMSO in solution are known to increase cell membrane fluidity (Gurtovenko et al., 2007), thus affecting the mechanotransduction apparatus. The effect of DMSO on platelet membrane fluidity is currently being studied using fluorescence polarization (FP) and dielectrophoresis (DEP) techniques at University of Arizona (unpublished data).

The toxicity of DMSO for human application has been largely investigated in literature. Studies performed in the 70's showed no significant abnormalities in extensive physical examinations or analyses of blood and urine during repeated applications of large amounts of DMSO to the skin of humans (Brobyn RD, 1975). Furthermore, Hull and colleagues found no DMSO-related effects in any of 38 humans, age 21-55, who received a topical application of an 80% DMSO gel in a single daily dose of 1 g/kg for 12 weeks (Hull et al., 1969). More recent investigations have instead proved that DMSO can be harmful or dangerous. Hanslick and co-workers performed a study in which they confirm the fact that DMSO in dosage of 0,3 ml/kg could produce apoptotic degeneration in developing mouse brain. This finding suggested that DMSO might produce similar damage in human children once exposed to DMSO during bone marrow transplantation (Hanslick et al., 2009).

A toxic effect of DMSO for platelets has been confirmed by the SEM investigation present in our study, where platelets treated with DMSO concentrations < 25% didn't display morphological changes in static conditions. On the other hand, when the percentages of DMSO in solution were > 40%, platelets appeared seriously damaged, with membrane lysed and intracellular compound clearly visible outside the platelets. More detailed toxicity studies should be carried out to verify possible sub lethal damages at lower concentrations.

To verify the ability of DMSO to protect platelets from physical forces, we subjected samples pre-incubated with different concentrations of DMSO to constant and dynamic shear stress profiles via the HSD. Exposure of samples to both 30 and 70 dyne/cm^2 for 5 min showed a dependency between platelet activation and the percentages of DMSO in solution. In fact, platelets treated with concentrations of DMSO < 15% presented a statistically significant reduction in platelet activation compared to control group. In our opinion, this phenomenon may be related to the ability of DMSO to increase membrane fluidity, thus helping platelets to better respond to shear stress exposure. Platelets treated with DMSO concentrations > 15% presented a level of activation similar to control group. This range of DMSO concentrations may have sensitized platelets by creating pores through the double layered lipid platelet membranes (Gurtovenko et al., 2007), thus increasing the platelet responsiveness to shear stress, which lead to an increased activation level detected with the PAS assay.

In addition, the versatility of the HSD allowed us to expose platelets to extremely dynamic shear stress profiles. The importance of this tests derived from the observation that changes of shear amount in MCS devices are rapid and variable. Moreover, platelets respond in different ways to constant or dynamic stimuli. This is due to the fact that platelets exposed to dynamic patterns of stimulation present a lower threshold of activation (Sheriff et al., 2013). We exposed platelets to a dynamic shear profile extracted from the PDF of the Debakey VAD corresponding to the 50th percentile of the total waveform. The results obtained confirm what established by the experiments under constant shear conditions, where concentrations of DMSO ≤ 15% protected platelet from shear-mediated activation. This shear stress profile extracted from CFD simulations within the DeBakey VAD was the same used in our previous study aimed at assessing the ability of commonly used antiplatelet agents to reduce platelet activation. Unlike the results obtained during the experimental campaign on antiplatelet drugs, where almost all the agents didn't offer a protection to platelets under the dynamic condition tested, data obtained in the present study showed a better behavior of DMSO as possible inhibitor of shear-mediated platelet activation.

The protection effects of DMSO were further investigated using the SCSD, a device able to subject platelets to shear stress peaks of thousands of dyne/cm^2 during experiments, as those experienced by platelets when passing through VADs. Data obtained showed significant differences between the PAS of control groups and samples treated with DMSO only during experiments at shear stress peaks > 680 dyne/cm^2. This can be related to the fact that during the experiments with exposure to lower shear peaks (< 680 dyne/cm^2) the minimum Stress Accumulation (SA) threshold needed for triggering platelet activation was not achieved, thus

in turn leading to a non-significant differentiation between control and samples treated with DMSO. Anyway, data obtained at 880 and 1440 dyne/cm^2 confirmed the ability of DMSO to protect platelet from activation after exposure to hyper shear.

As already mentioned, samples exposed to shear conditions via SCSD were not subjected to the same SA as within HSD (Table 4.3 A-B). In an attempt to compare the results obtained with the two devices and to assess the behavior of DMSO after exposure to hyper shear stress for a prolonged period of time, we increased the number of loops performed with the SCSD. This allowed to compare the SA experienced by platelets at 30 dyne/cm^2 for 5 min in the HSD with the one within SCSD at 880 dyne/cm^2 for 21.5 s of actual exposure to hyper shear. The results obtained showed that the average platelet activity state of the control groups obtained with SCSD was higher compared to the control of the HSD experiments. In both cases samples treated with 5% DMSO displayed a significant reduction in PAS compared to their controls. Interesting considerations arise from the results obtained. First, it was possible to compare the results obtained with two different devices (HSD end SCSD) and to demonstrate a similar behavior of DMSO in the two configurations tested. Furthermore, the assumption reported in the literature that dynamic shear stress exposure has a larger impact on platelets activation was hereby confirmed (Sheriff et al., 2013). In fact, it is worth to mention that the level of activation detected for platelelts exposed to 30 dyne/cm^2 for 5 min within the HSD was comparable with the one detected for platelets exposed to shear peaks of 880 dyne/cm^2 in the SCSD for shorter exposure times, like the ones characterizing the first experimental campaign with SCSD (Figure 4.13). In this case, although the level of platelets activation was comparable between samples exposed to a constant shear pattern (HSD) or a dynamic shear pattern (SCSD), the TSA experienced by platelets at constant shear was exceptionally higher with the respect to the levels obtained at dynamic conditions (9000 vs 1776 dyne s/cm^2), thus indicating how platelets are more sensitive to variable shear stress patterns compared to constant ones.

To check whether the reduction in the thrombin release detected in samples treated with DMSO was due to the protective effect of chemical compound or caused by a reduction in platelets function, we verified the viability of platelets after exposure in the SCSD. Samples incubated with DMSO and exposed to shear were further incubated with arachidonic acid with the aim to assess that, even after continuative exposure to DMSO and physical forces, platelets may adequately respond to chemical stimuli. Results obtained showed a normal platelets reactivity to the chemical agonist, thus confirming the viability of cells. It was also

proved that DMSO provides a good protection against platelets activation driven by physical forces, but it has very little effect when the activation is mediated by a chemical process.

Besides the results obtained, further investigation need to be carried out to confirm the ability of DMSO to protect platelet exposed to shear stress. Moreover, we think that other chemical compounds should be tested to assess their ability in protecting platelets when passing though cardiac devices. Among them, non-toxic polar lipid compounds may be able to penetrate platelet membranes, thus increasing their fluidity. Our analysis indicates that a paradigm shift is required in the development of new antiplatelet agents for the treatment of shear mediated platelet activation with respect to the conventional approach to the treatment of chemical-mediated activation.

We believe that the discovery of new compounds able to affect the mechanotransduction apparatus will potentially reduce the need of large antithrombotic therapies in the use of VADs and MCS devices, offering an effective protection to platelets when exposed to high shear stress conditions.

5

Prospective applications of Platelet Activity State (PAS) assay: from in vitro investigation of ventricular assist devices to the possibility of clinical use

5.1 Introduction

The development of mechanical circulatory support (MCS) devices, i.e. ventricular assist devices (VADs) and total artificial hearts (TAHs), offers a valid solution for heart failure (HF) treatment, either as a bridge or as an alternative treatment to heart transplantation (Copeland et al., 2004). However, current devices have many limitations, including the propensity for thrombosis and thromboembolism. These adverse events often introduce clinical post-operative complications that further increase the healthcare burden for the patient and society. Recipients of VADs and mechanical heart valves (MHVs) are always faced with the requirement of a lifetime complex and risky anticoagulation regimen. Although the current generation of MCS devices shows a vast improvement in hemodynamic performance over their predecessors, reducing flow-induced thrombogenicity still remains a major challenge. Such important goal may be achieved through the development of new generation devices that are optimized to be thromboresistant (i.e., with a reduced need for antithrombotic therapy). Nevertheless, hemodynamic studies employed for device optimization during the R&D stage have no iterative synergy between numerical and experimental methods, thus leading to a significant limitation regarding device thromboresistance optimization.

The DTE method, deeply described in both Chapter 2 and Chapter 3, tries to overcome these limitations in the optimization of MCS devices thrombogenicity. However, the ability to investigate the devices of interest not only *in silico* and *in vitro* with tests performed in bench shearing devices, but also *in vivo*, within animal models or clinical trials, is mandatory to certify the improvement brought by new devices, developed with the goal of reducing the need of lifelong antithrombotic therapies (Figure 5.1).

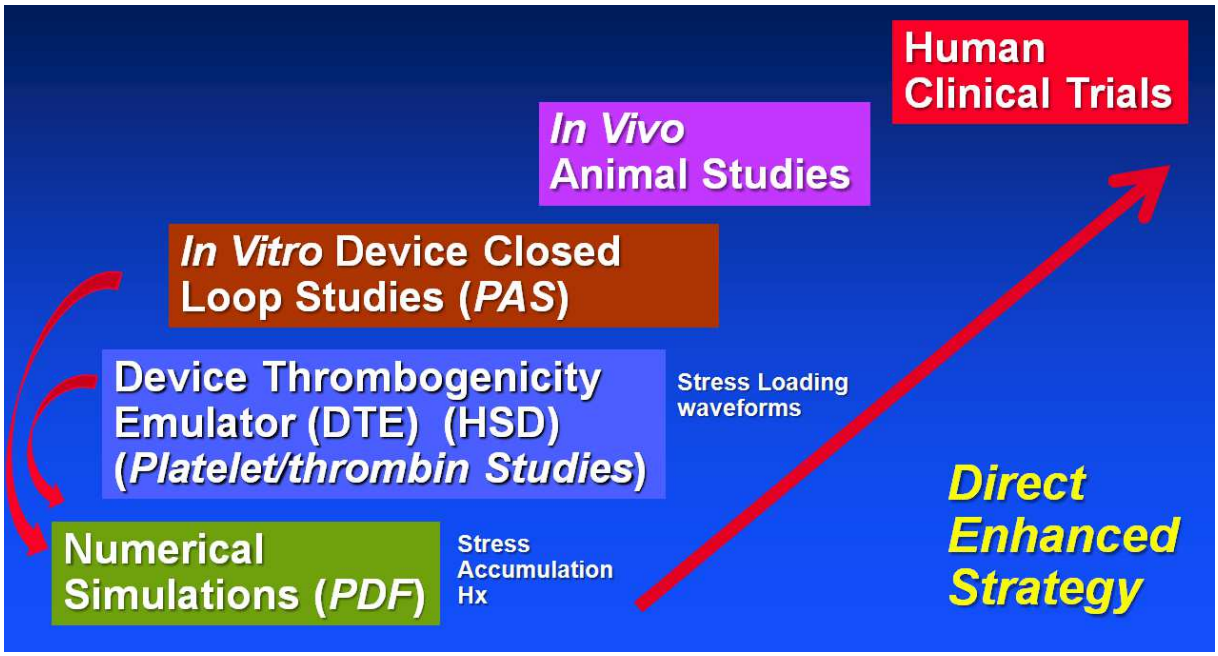

Figure 5.1. The importance of DTE method in the strategy of optimizing cardiac devices. The possibility of investigating the thrombogenicity of VADs with both CDF simulations and *in vitro* studies may allow to avoid useless in vivo campaign with devices that are not optimized.

In this chapter, a more hybrid approach will be presented to investigate actual VADs both *in vitro* and *in vivo*. In fact, a hypothetical connection line will be traced among the reported investigations, in the attempt to retrace the phases enlightened in Figure 5.1. The results obtained and presented hereinafter are still not complete, but permit to draft preliminary considerations on the utility of PAS also for clinical use.

In particular, **the first study consisted of an *in vitro* investigation of two VADs (Debakey VAD and HeartAssist 5) in appropriate bench loops, using both gel-filtered platelets (GFP) and whole blood (WB) as experimental fluids.**

Instead of using shearing profiles extracted from CFD simulation and tested via HSD, we characterized the thrombogenicity of real commercialized devices in flow loops. Moreover, the study allowed to validate the PAS assay behavior with GFP compared to WB.

The HeartAssist 5 (HA5) was further investigated with an *in vivo* campaign on calves, where the VAD was implanted and its effect on platelet activation was monitored using PAS assay for a total of 2 hours after implant. This investigation tried to assess the ability of PAS assay to detect changes in platelet activity state during animal studies, which are mandatory during the characterization phase of cardiac devices.

So far, to the best of our knowledge, the only attempt to connect the PAS assay to clinical relevant issues was described in the study of Ramachandran and colleagues, in collaboration with Stony Brook University, in which the effect of nicotine on platelet

activation was assessed (Ramachandran et al., 2004). Despite being preliminary, the studies here described represent a further application of PAS assay for clinical purposes.

5.2 *In vitro* comparison between DeBakey VAD and HA5

5.2.1 Mock loops for the study of VADs

In vitro evaluation of VADs is of vital importance during both the prototypal and post-production phases, as well as during and after the introduction of new and updated versions of previously developed devices. This is the main reason why several research groups have created mock loops for the fluid dynamic characterization of VADs (Schampaert et al., 2014; Timms et al., 2005a-b; Pantalos et al., 2004).

In particular, Schampaert and colleagues developed an integrated mock circulation system able to reproduce physiological fluid dynamic conditions for testing cardiovascular devices. The model included a systemic, pulmonary, and coronary circulation, as well as an elaborate heart contraction and a realistic heart rate control models (Figure 5.2).

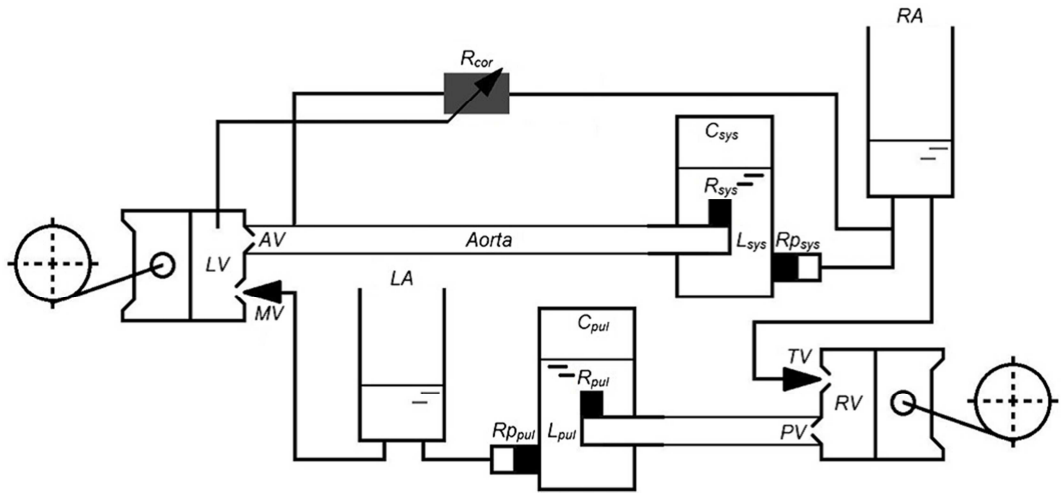

Figure 5.2. Schematic representation of the *in vitro* mock circulation model proposed by Schampaert et al., (2014) including a systemic, pulmonary and coronary vascular bed.

Authors tested their system in response to changes in left ventricular contractile states, loading conditions and heart rate. Such versatile bench simulators are extremely useful especially when characterizing VADs, since the strong interaction between these devices and the ventricle cannot be properly modeled without the appropriate response of the heart and circulation. The possibility to reproduce baroreflex control allowed to mimic the contractile properties of the heart under pathological conditions. Moreover, a simple lead-lag controller proved to be capable of mimicking left ventricular function, enabling realistic evaluation of cardiovascular devices in phantom studies (Schampaert et al., 2014).

A different approach was followed by Timms and colleagues. They described the realization of a complete and compact mock loop for testing cardiac devices, with a particular feasibility for the investigation of VADs (Timms et al., 2005b). Indeed, the author showed that the different pumps inserted into a complete systemic and pulmonary mock circulation rig provided suitable non-pulsatile or simulated pulsatile left heart failure environments (Timms et al., 2005a). The LVAD pump investigated in the study demonstrated the ability to re-establish hemodynamic parameters of pressure and perfusion, returning from pathological to normal conditions. One of the most prominent characteristics of the mock loop presented by Timms and co-workers was the ability to easily perform transition between physiological and pathological states, representing both conditions of rest and heart failure, furthermore instantly recording volume changes due to fluid dynamic conditions tested (Timms et al., 2011b) (Figure 5.3).

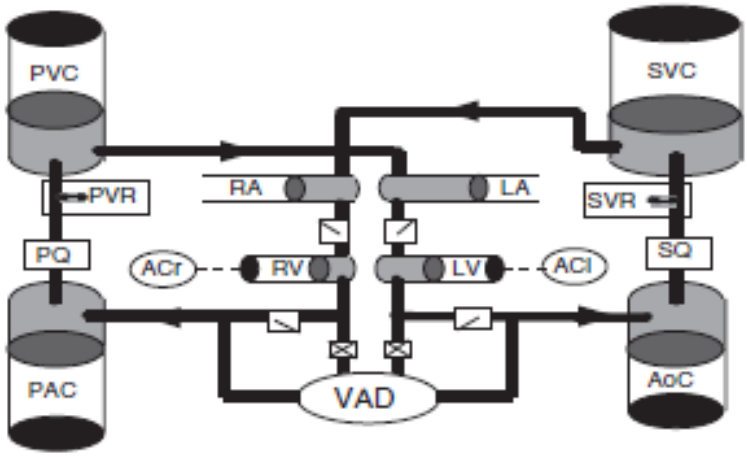

Figure 5.3. Schematic representation of mock circulation loop developed by Timms et al. (2005a). ACl, left air compressor; ACr, right air compressor; AoC, aortic compliance; LA, left atrium; LV, left ventricle; PAC, pulmonary arterial compliance; PQ, pulmonary flow rate; PVC, pulmonary venous compliance; PVR, pulmonary vascular resistance; RA, right atrium; RV, right ventricle; SVC, systemic venous compliance; SVR, systemic vascular resistance; SQ, systematic flow rate; VAD, ventricular assist device.

Although these mock loops mimic the physiological environment with high precision, their complexity makes them not suitable for studying specifically VAD-related platelet activation.

Up to now, just a few *in vitro* studies have been performed to investigate the impact of the MSC devices in terms of platelet activation.

Voorhees and colleagues reported that platelet damage in extracorporeal circuits is mainly due to the interaction between biological fluids and foreign surfaces within devices, as well as non-physiological shear stress conditions. Their method allowed to assign a numerical score to platelet damage assessed by direct visualization via phase contrast microscopy. Authors

observed the influence of materials and hemodynamic design on platelet damage, thereby allowing evaluation of the impact of different cardiac devices (Voorhees et al., 1990).

As detailed explained in Paragraph 3.3.5, the attention on MCS related platelet activation was increased by the development in Stony Brook University of a chemical assay, dubbed Platelet Activity State (PAS) assay, which allowed a series of *in vitro* investigations on VADs or other biomedical devices, where platelets were subjected to mechanical or biochemical stimuli and their activation was detected by the assay (Jesty et al., 1999). In 2003, Jesty and co-workers circulated human platelets within loops where exposure to shear stress was adjusted by independently varying flow rate, viscosity, and time of shear exposure. The study confirmed that low-level platelet activation caused by shear stress in a circulation loop may be quantitatively assessed using PAS assay, but more importantly it emerged that exposure of platelets to shear conditions of the same order as found in the vasculature causes significant platelet activation, opening the way to possible further applications of PAS assay to the study of more subtle or less traumatic cases involving shear-mediated activation (Jesty et al., 2003).

More recently, an *in vitro* study conducted by Sheriff compared aspirin solutions created in lab for *in vitro* tests with its metabolized version obtained from human volunteers in terms of their efficacy on platelets subjected to shear stress exposure (Sheriff et al., 2014). In the study, purified human platelets were circulated for 30 min in a flow loop containing the DeBakey VAD. The results proved that *in vitro* treatment with antiplatelet drugs such as aspirin is as effective as *in vivo* metabolized aspirin in testing the effect of reducing shear-induced platelet activation in the VAD (Sheriff et al., 2014).

Except from (Voorhees et al., 1990), all the above studies on platelet activation were conducted using GFP**.** No studies of different VADs involving WB were performed in flow loops, using the PAS assay as a method to assess platelet activation. To compensate this methodological lack, **we decided to realize two sets of experiments that used both GFP and WB. Samples were circulated within already characterized flow loops (Sheriff et al., 2014) with both DeBakey VAD and HA5 and the platelet activity state was quantitatively assessed using the PAS assay.**

5.2.2 Experimental Concept

The experimental concept of the study is described in Figure 5.4. Further details are provided in paragraph 5.2.3.

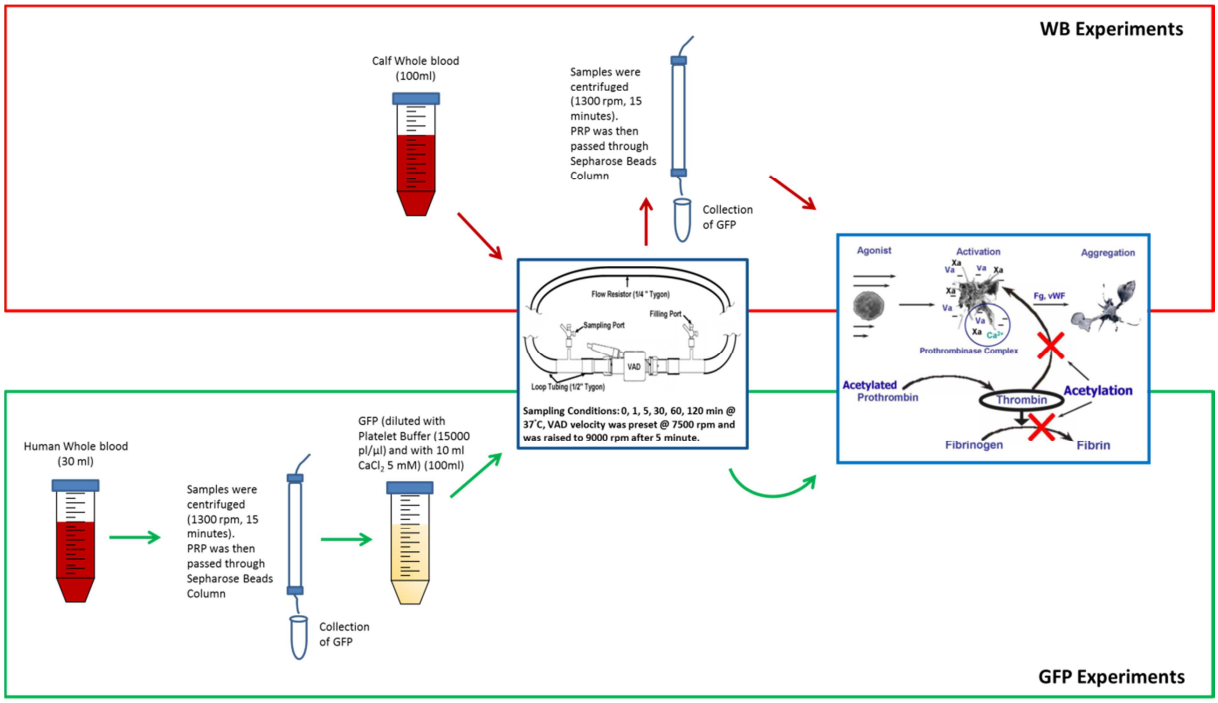

Figure 5.4. Experimental Concept. WB or GFP solutions were used in flow loops with DeBakey VAD and HA5, in order to assess devices thrombogenicity over a total period of time of 120 min. Adapted from (Girdhar et al., 2012; Sheriff et al., 2014).

5.2.3 Experimental protocols

The experiments were conducted as follows. Both WB and GFP were tested using appropriate flow loops, within which Debakey VAD and HeartAssist 5 (HA5) were used in order to shear samples.

WB was obtained from an abattoir in collaboration with University of Arizona, one hour prior to the experiments. A total volume of 500 ml of calf blood was collected into 3.8% trisodium citrate (9:1,v/v) for each experiment. GFP was obtained from 30 ml of human blood, which was drawn from the antecubital vein under consent from the subjects and the Institutional Review Board (IRB) of University of Arizona, following the protocol already described in Paragraph 3.3.2. The choice of calves instead of humans for the experiments with WB derived from the excessive amount of blood needed for each experiment, which made not possible to perform loop studies with human WB. On the other hand, it was interesting to

investigate human GFP using the same loops in order to compare data obtained in a previous study performed in the same conditions at Stony Brook University (Girdhar et al., 2012).

For each flow loop, the total volume of either WB or diluted GFP in buffer solution used was 100 ml. GFP or WB mixtures were exposed for a total time of 120 min at 37°C in a flow loop containing a DeBakey VAD (MicroMed Cardiovascular Inc., Houston, TX) or its DTE-optimized version, the HeartAssist 5 (HA5, MicroMed Cardiovascular Inc., Houston, TX). VADs inlet and outlet ports were connected to short 0.5" Internal Diameter (ID) Tygon R3603 loop tubing and a 0.25" ID Tygon R3603 flow resistor tubing of 47" length (Fig. 5.4, center box. Girdhar et al., 2012; Sheriff et al., 2014). VAD pump conditions were set at 4 l/min of cardiac output and 9000 rpm, corresponding to average physiological and clinical operating ranges, and they were controlled using the MicroMed Clinical Data Acquisition System (CDAS). These settings corresponded to a pressure rise of ~ 70-80 mmHg across the pump (Sheriff et al., 2014). Platelets were taken at t = 0, 5, 10, 30, 60 and 120 min for the Platelet Activation State (PAS) assay (Jesty et al., 1999) through a silicone sampling port upstream of the VAD. To study platelet function in devices, it was mandatory to use an assay where platelet function in terms of thrombin generation can be measured as a direct function of shear stress alone, as PAS assay. For each VAD experiment, the obtained values were normalized to maximum activation obtained by sonication (Schulz-Heik et al., 2005). The Platelet Activation Rate (PAR) was calculated from the slopes of best-fit lines of normalized PAS values.

WB obtained from calves was used in loops without any dilution. 15 ml of blood were drawn at each time point. This sampling volume was due to the fact that WB needed to be filtered each time through Gel-Sepharose Chromatography columns to obtain GFP, necessary to correctly perform the PAS assay. The minimum amount of blood sample that was considered enough was thus 15 ml.

To avoid depression within the loop at the moment of sampling for the assay, each volume of WB was substituted by platelet-poor plasma (PPP), obtained centrifuging WB at 4000 rpm for 20 min.

On the other hand, human GFP used for the experiments was equilibrated to a final concentration of 15000 pl/µl in platelet buffer consisting of HEPES-modified Ca2+-free Tyrode's buffer containing 0.1% fatty-acid-free bovine serum albumin (Neuenschwander et al., 1988). We decided to dilute GFP because of the limitation in platelet concentration and volume obtainable from human donors, since IRB approval allowed to drain a maximum of

30 ml of blood at each donation. The volume of GFP drained at each time point was 2 ml and it was substituted with platelet buffer in the loop.

In order to compare data obtained from GFP with WB loops, the amount of platelets analyzed during PAS assay at each time point was 5000 pl/µl. This removed the discrepancy between the two different concentrations of platelets during loops, i.e. 200-300 x 10^3 pl/µl for WB and 15 x 10^3 pl/µl for GFP experiments.

5.2.4 Results

The results obtained with WB (Figure 5.5 - A) and GFP (Figure 5.5 - B) are represented in Figure 5.5.

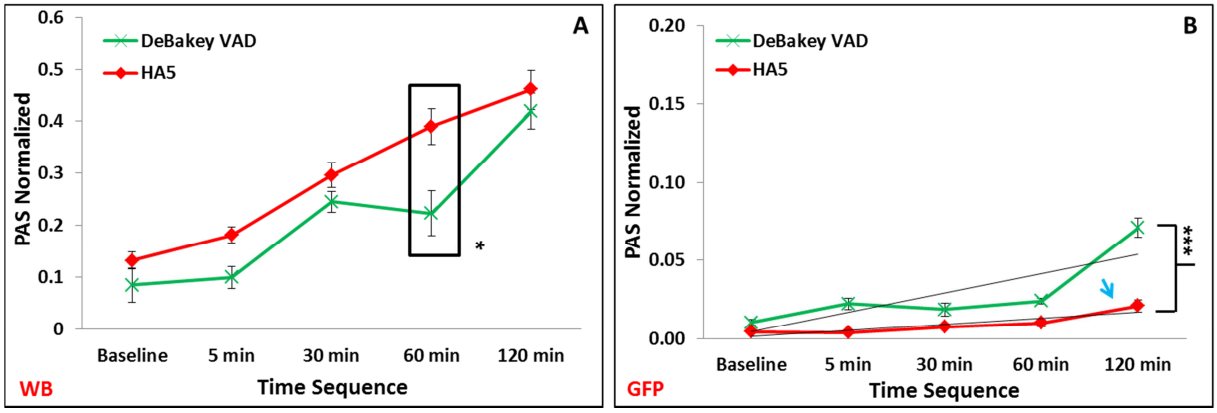

Figure 5.5. *In vitro* flow loop. WB (A) and GFP (B) were recirculated in flow loops with DeBakey VAD and HA5. Experiments were conducted for a total exposure time of 120 min. N Expt = 6+ from at least 3 different blood samples. Data are represented as mean ± SEM. * $p < 0.05$. *** $p < 0.0001$.

The experiments performed with WB showed no differences in the platelet activation trend between the two devices investigated, even if there was a statistical significant reduction in the platelet activity state of samples tested with DeBakey VAD compared to the HA5 after 60 min of experiment. Nonetheless, this behavior was not found at 120 min.

Data obtained with GFP showed instead a different activation trend between the two VADs. In fact, despite PAS values of the experiments with GFP were lower compared to the ones obtained with WB, we found that the platelet activation level provided by the HA5 was significantly reduced with the respect of the DeBakey VAD, after 120 min of experiment ($p < 0.0001$).

5.2.5 Observations

Recently, Girdhar and co-workers used the DTE methodology to compare two rotary LVADs (DeBakey VAD vs HA5, Micromed Houston, TX) under clinical conditions in flow loops with GFP. Their primary aim was to demonstrate that HA5, which was an optimized version of the DeBakey VAD developed using engineering re-design modifications, had a less thrombogenicity impact compared to its predecessor (Girdhar et al., 2012). The results obtained showed that platelet activity rate for the optimized device was an order of magnitude lower.

In our study, we compared the same devices (DeBakey VAD vs HA5), but in flow loops containing either GFP or WB. The comparison results varied between the two experimental conditions. In fact, when the WB was used, significant differences between DeBakey VAD and HA5 ($p < 0.05$) emerged only after 60 min of exposure, with a reduced platelet activation detected during the experiment with the former VAD. This reduction was not detected at 120 min. The comparison between VADs performed with calves blood reported a higher platelet activity state compared to GFP loops (0.5 vs 0.05 at 120 min). Nevertheless, no significant differences in terms of platelet activity rate (PAR) calculated between 0 – 120 min were found between DeBakey VAD and HA5.

The results obtained with GFP confirmed the ones obtained by Stony Brook University (Girdhar et al., 2012; Sheriff et al., 2014) and contradicted the results of WB experiments, since a better behavior of HA5 compared to DeBakey VAD emerged in terms of both PAR (0.0039 vs 0.0122 min^{-1}) and PAS values at 120 min (0.02 vs 0.07, $p < 0.0001$).
Additional considerations regarding the results obtained are reported in paragraph 5.4.

5.3 *In vivo* assessment of the thrombogenicity of HA5 in bovine model

5.3.1 Animal study for VADs validation

In the process of development of new MCS devices, large animal trials allow to test devices in a physiologic environment mimicking the final destination in humans. In fact, the interaction of mechanical parts with biological fluids needs to be investigated not only *per se*, but also in the presence of the reactivity of the whole organism. This, for instance, is key to provide information about possible harmful inflammatory reactions that in turn may lead to thrombi formation and pump damage.

In the last decades, smaller and less invasive pumps have been developed with the objective of overcoming limitations of previous devices and animal tests have been performed during their whole evaluation processes.

HeartWare developed a miniaturized passive magnetically suspended centrifugal VAD. Core and colleagues evaluated the new pump design in a series of 22 animal experiments, for a total of 618 cumulative implant days (Core et al., 2004). The animal studies were conducted for a variety of purposes, including anatomical fit, design iteration and confirmation of hemocompatibility. In particular, five long term sheep implants led to the final pump design. All experiments were conducted over a mean period of 83 ± 12 days without anticoagulation. Animals were electively terminated after 90 days except one, which had an electrical failure in a controller prototype. Overall pump performance at a rotational speed of 3000 revolutions per minute (rpm) was excellent, with a mean pump flow rate of 6.1 ± 1.4 l/min. The whole flow range investigated was from 3 to 11 l/min. During the study, no pump thrombus formation or pump failures occurred. Animals necropsy revealed clean pumps and no signs of pump related end-organ damage. Initial animal experiments demonstrated excellent performance of the proprietary HeartWare pump technology. Based upon these results, pump design was finalized and now the HVAD has been implanted in more than 2000 patients worldwide.

HeartWare HVAD was also investigated by Tuzun and co-workers. Devices hemocompatibility and long term end-organ effects were evaluated in 6 healthy sheep. The implant duration was 90 days; after that, animals were sacrificed. Hematologic and biochemical tests of liver and kidney functions were performed, both preoperatively and throughout the study. During the 90 days, HVAD showed exceptional hemocompatibility and reliability, with no significant complications (Tuzun et al., 2007). After the first human

implant performed in 2006, HVAD underwent other animal trials in order to confirm its feasibility and reliability, as well as to assess the behavior of newer versions developed. Slaughter and other clinicians have implanted an HVAD in a calf to monitor hemodynamic and device's performance over 30 days (Slaughter et al., 2009). This study confirmed that no mechanical failure and thrombi formation occurred upon explantation of the VAD. The good results obtained underlined the HVAD beneficial effects, such as the increased availability to a broader patient population, the lower risk of infection, simplified implantation procedures and improved durability.

As emerged above, animal studies are an essential step not only during the process of new cardiac device manufacturing, but also in the optimization of VAD previous version, because they provide key information for obtaining better clinical outcomes.

Concerning the DeBakey VAD (MicroMed Cardiovascular Inc., Houston, TX), extremely precise CFD simulations, as well as re-design of limited VAD parts, allowed to modify the original device, thus obtaining its optimized version, the HeartAssist 5 (HA5, MicroMed Cardiovascular Inc., Houston, TX). The previous DeBakey pump was made of titanium; rpm ranged from 7,500 to 12,500, with flow rates up to 10 l/min. The first human implant of the MicroMed DeBakey VAD was performed in Berlin in November 1998. Up to 2010, there have been 451 implants (155 of those in the U.S.) of DeBakey VAD, including 19 pediatric (Noon et al., 2010). Because of sporadic cases when pump thrombi formation was evidenced, some changes have been incorporated into the design over the years, in regard to the prevention of adverse events. The improvements resulting from multiple iterations of the pump have been incorporated into the new design, the HA5. After these pump modifications, animal tests in calves were performed without anticoagulation. The new device was implanted in 10 calves, each implant lasting 60 days. In these animal tests, authors have not encountered any thrombus formation (Noon et al., 2010). The thrombogenicity of the DeBakey VAD and its optimized version, the HA5, were further characterized using the DTE technology by means of both simulations and *in vitro* experiments in which the platelet activity state was detected using the PAS assay (Girdhar et al., 2012, Sheriff et al., 2014). Since the latter was never used to assess the platelet activation level of the above mentioned VADs (DeBakey VAD and HA5) on animal studies, **in the present section we investigated the thrombogenicity of the HA5 using the PAS assay within an *in vivo* campaign on calves, allowing also to verify the effectiveness of using this assay for *in vivo* application. The integration of the results obtainable from *in vivo* campaign and *in vitro* studies may offer**

additional information for a better characterization of the thrombogenicity of current and future cardiac assist devices.

5.3.2 Experimental Concept

The experimental concept of the study is described in Figure 5.6. Further details are provided in Paragraph 5.3.3.

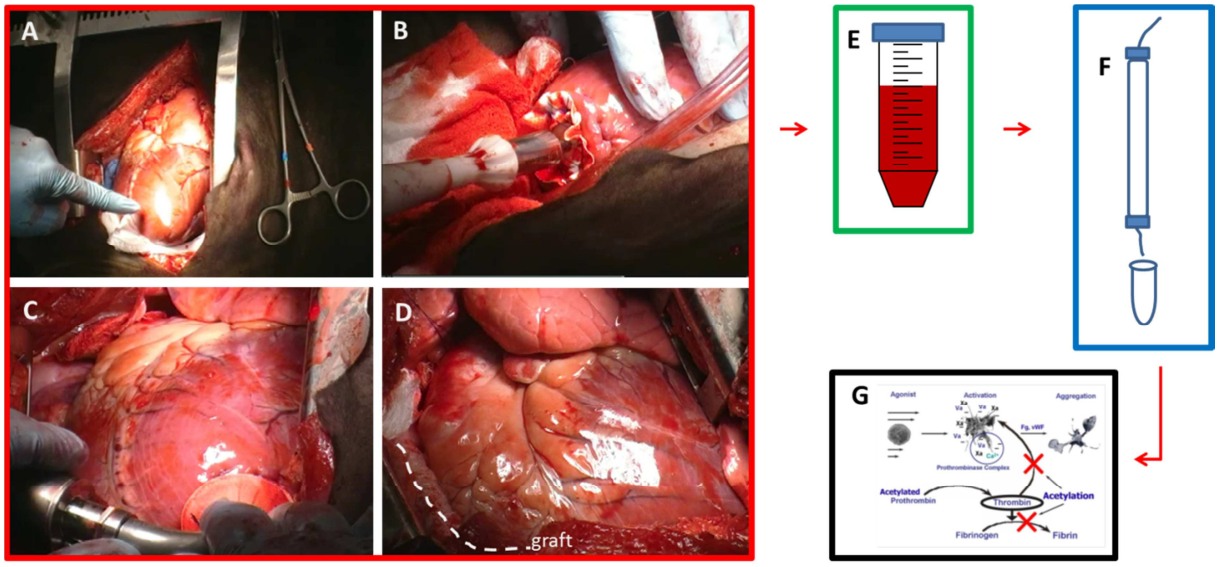

Figure 5.6. Experimental Concept. Surgical procedure for the implant of the VAD was characterized as follows: (A) Calf heart apex exposure, (B) creation of a hole in the apex for VAD inflow insertion, (C) connection of the LVAD to the apex, (D) Successful implantation of the LVAD, with the graft correctly connected to the outflow of the pump and anastomosed to the aorta. After VADs implant, blood samples (E) were taken at 0, 5, 30, 60 and 120 min and GFP was obtained after Gel-filtration (F). Platelet activity State was assessed through PAS assay (G).

5.3.3 Experimental Protocols

All surgery procedures were performed at the University Animal Care Facility (UACF) of the Arizona Health Science Center (AHSC). In particular, four HA5 were implanted in four young adult calves (115 ± 25 Kg).

In the preparation room, each calf was placed in head catch restraint and pre-medicated with xylazine (0.1-0.2 mg/kg) and butorphanol (0.01-0.02 mg/kg). Once sedation was achieved and cannulation was performed to monitor the vital parameters during surgery, the animal was considered ready for the procedure. Standard left intercostal thoracotomy was performed between the 4th and 5th rib by surgeons and/or UACF veterinarian. After exposure of the heart, surgeons implanted the LVAD on the apex of the left ventricle. During the

surgery, the animal was heparinized at 150 units/kg to maintain an Activated Clotting Time (ACT) > 250 seconds. At the end of the implant, the pump was turned on at low speed (7500 rpm) first. After verifying that the pump was functioning, with no leakages and kinks, VAD velocity was increased at 9000 rpm, typically corresponding to a flow of approximately 4 l/min. 20 ml of blood were sampled from the animal before sedation, as well as at 5, 30, 60 and 120 min after starting the pump. Blood obtained at each time point was centrifuged at 1300 rpm for 15 min and GFP were extracted following the protocol described in Paragraph 3.3.2. After 120 min from implant of the device, animals were euthanized by UAC veterinary staff. In particular, calves were heavily sedated with Ketamine (3-4 mg/kg) to ensure recumbency and then treated with a barbiturate overdose (Beuthanasia-D or similar, 0,22 ml/kg).

This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona.

5.3.4 Results

The platelet activity state (PAS) values at different time points after the implant of HA5 (Figure 5.7 A-D) in calves are reported hereinafter.

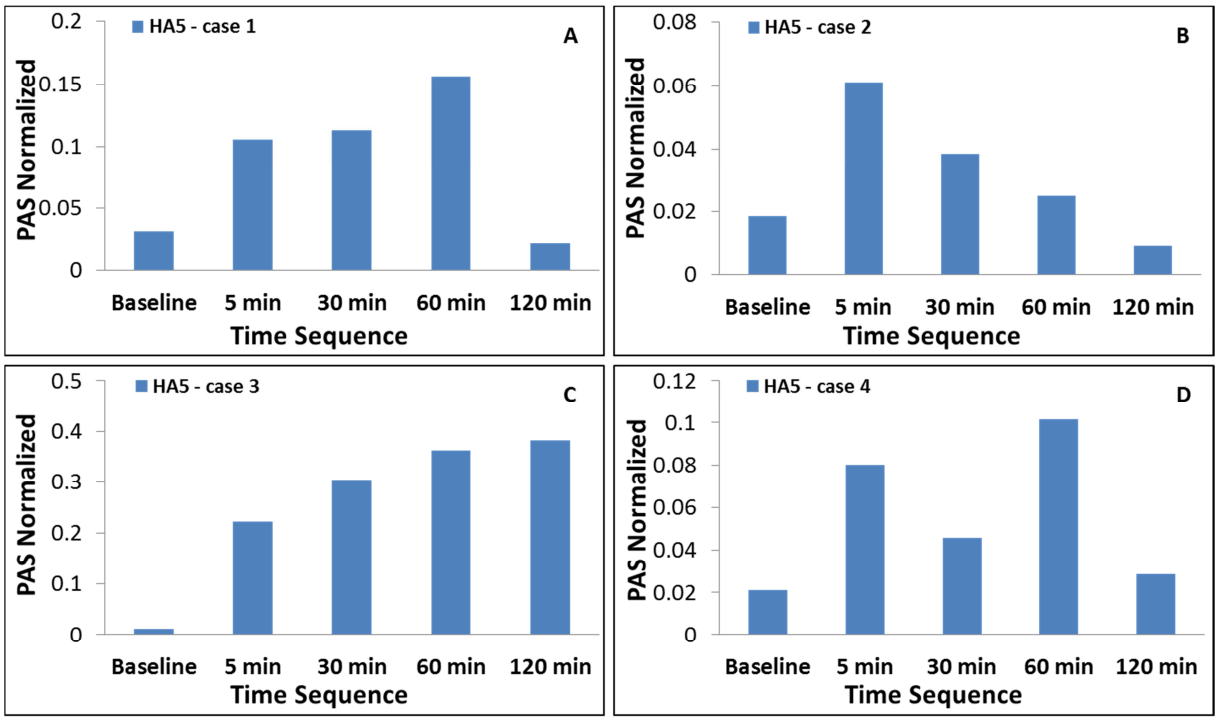

Figure 5.7. A - D. Platelet activity state *in vivo* after exposure to HA5. The VAD was implanted in 4 calves (case 1 - 4) and the results obtained in each case are represented in term of PAS values.

We decided to represent the results obtained by showing each single PAS trend. Data obtained seemed different among all cases. However, some common considerations regarding the whole pool of experiments can be drawn. First, an increase in platelet activation was detected for all cases after the VAD implant compared to the baselines, thus pointing out the ability of PAS assay to detect an abrupt or isolated event that can cause platelet activation, like the initial blood passages through a VAD.

However, this platelet activation cannot be certainly attributed to the VAD impact. More likely, in fact, the discrepancy between the level of activation detected before the sedation of the animal and the level after 5 min of VAD implant may be due to the operative stresses to which the animal was subjected during surgery. Indeed, no repeatable PAS trends were found between 5 and 60 min after the device implantation, where PAS seemed increasing or decreasing depending on the specific case. Interestingly, after 120 min, a platelet reactivity reduction was detected by PAS assay in three out of four cases. A possible explanation regards the occurrence of some mechanism of platelet activation inhibition provided by the animal organism over time. In fact, except for one case (Figure 5.7 - C), PAS values at 120 min resulted similar to the ones obtained when the calves blood was tested before sedation (baseline condition).

Unfortunately, the obtained results did not allow any consideration about the thrombogenicity of the device investigated. Indeed, the duration of the experiment was too limited for systematically characterizing the effects of different devices on platelet activation. Further information may arise from the performance of long term chronic studies, during which the effect of VADs could be assessed for a prolonged period of time.

5.4 Discussion

In the present chapter, we investigated the ability of PAS assay to detect platelet activation after exposure to shear stress in both *in vitro* flow loops or animal VAD implants.

The results obtained showed preliminary trends that allowed us to make hypotheses that need to be further verified. The *in vitro* experimental campaign confirmed that PAS assay has a better sensitivity when testing platelets alone rather than WB. In fact, the presence of a myriad of other elements in blood, together with the experimental condition tested, in which a small amount of blood (100 ml) was recirculated within VADs at high speed rate (9000 rpm, 4,5 l/min), may have led platelets to activate anyway, thus masking the effect of device modification in HA5.

Furthermore, during the experiments with WB, the positive activation feedback due to the prothrombinase complex (factor Xa, Va, Ca^{2+}, anionic phospholipids) was not inhibited. On the contrary, the use of purified platelets (GFP) avoided this possible mechanism of activation. This phenomenon may have increased platelet activation, thus resulting in a higher PAS level detected with the assay during the experiment with WB with respect to the ones with GFP.

As mentioned in paragraph 2.6, there are other factors that can explain the differences between WB and GFP experiments (Zhao et al., 2007). The hematocrit (Ht) and viscosity values in WB may have increased the overall mechanical stress exerted on platelets; in addition, a platelet margination effect may have been included (Du et al., 2014). In the presence of RBCs, platelets exposed to laminar shear are marginated towards the outer edges of the tubing constituting the circuit, thus being exposed to higher wall shear stress regimens compared to the experiments with GFP. This would partially explain the higher platelet activation values we found during WB experiments. Moreover, it should be mentioned that platelet concentration during WB experiments was more than 10 times higher compared to GFP studies (100-300x10^3 vs 20x10^3 pl/µl). Although we tried to minimize this effect by standardizing platelet concentration during the PAS assay (5000 pl/ µl), an uncontrolled contribution related to platelet concentration may have been present.

The *in vivo* animal studies pointed out even a stronger variability between results. All the animal cases showed an elevated platelet activation state after the VAD implant. This may be related to the sensitivity of calf blood to surgical procedures. Such phenomenon has been elucidated in sham studies. Snyder and colleagues evaluated platelet behavior in 8 calves undergoing a sham surgical procedure, thus allowing the separation of surgical effects of

VAD placement from blood contact with the operating device (Snyder et al., 2002). The invasive nature of the implantation procedure, including coring the left ventricle and creating the aortic anastomosis, produced platelet activation in the early postoperative period. The data collected from the surgical sham procedure on calves clearly demonstrated a significant increase in all of the activated platelet indicators after the operations and a return to preoperative values only after 17 days. These results clarify the substantial early contribution to platelet activation induced by the implantation procedure, demonstrating the need to be cautious when attributing increases in activated platelets to device operation in this early postoperative period (Snyder et al., 2002).

Comparing the results obtained *in vivo* and *in vitro*, it emerged that during the bench investigation the level of platelet activation was higher compared to the *in vivo* data. However, this discrepancy was expected because the total volume of blood recirculated during the loop assessments was lower (100 ml) compared to the animal model (≈ 5 l), thus intrinsically magnifying the effects exerted on each individual particle.

Finally, the use of WB has a procedural drawback consisting in the delay between the sample extraction and the performance of PAS assay. In fact, the PAS assay can test only purified platelets, which forced us to filter each blood samples before measuring thrombin release. This delay between blood sampling and performance of the assay may have further affected the platelet activity state of the tested samples.

The results reported in the present chapter showed that PAS assay can be used to assess platelet activation starting from either WB or GFP. Nonetheless, the ability to detect slight variations of platelet activity state obtainable using GFP suggested that the latter is preferable. The results obtained with WB pointed out the capacity of PAS assay to detect marked differences of activation, but not to offer the reliability needed in experimental campaigns for VAD assessment. Nonetheless, we believe that future minor modification of the PAS assay might make it more reliable when used in association with WB, becoming suitable for clinical applications. To further investigate the use of the PAS assay as clinical asset, we started a pilot study on three patients that underwent mechanical circulatory support devices implantation (HeartWare HVAD, Thoratec HeartMate II and Syncardia TAH). Their platelet activity state was monitored via PAS assay for a total duration of three days after the surgery. The obtained results are reported in the Appendix. Even if very preliminary, this study demonstrated a good feasibility of the PAS assay for detecting platelet activation from MCS patients' blood. In the future, we envision the use of a modified PAS assay for the assessment

of MCS patient platelets activation level after implant, thus providing a real time monitoring of the device behavior for a safer control of the patients' blood condition.

6

Conclusive remarks

6.1 Main Findings

Mechanical circulatory support (MCS) devices, the most prominent solution for treatment of heart failure (HF), are still burdened with several post-implant complications like pump failure, thrombotic events, infections or emboli formation. In the attempt to reduce both thrombosis and other clotting disorders, several antiplatelet agents have been developed in the last decades (Simon et al., 2008). Although such agents have been extensively tested in static conditions, up to now their effectiveness under shear stress, i.e. in relation to physical forces encountered when the blood passes thorough VADs, has been only marginally investigated. One of the methods developed to reproduce *in vitro* the fluid dynamic conditions encountered *in vivo* is the DTE method (Girdhar et al., 2012). Testing the effects of several chemical agents on platelets through a platform (HSD) able to reproduce with high fidelity the shear stress profiles that blood encounters within VADs is extremely important, since it provides a comprehensive view of the pharmacological treatment. The DTE method in combination with the PAS assay, which is able to determine a one-to-one relation between thrombin formation and platelet activity state, represents an extremely strong tool to assess the thrombogenicity of different cardiac devices and to test new solutions aimed at reducing the need of antithrombotic therapies.

In this scenario, we focused on the assessment of the ability of pharmacological treatments to reduce MCS devices thrombogenicity. We thus conducted a systematic campaign of *in vitro* experiments to provide a comprehensive characterization of the interaction between those treatments and the exposure of platelets to shear stress conditions. Within this complete *in vitro* study, the platelet activity state was monitored using a specific chemical procedure, the PAS assay. With the vision of extending the use of this assay for further clinical diagnostic applications, we conducted also a set of preliminary studies *in vitro* and *in vivo* within animal models.

Among the pharmacological approaches developed to face the pathological interaction between platelets and MCS devices, we studied a set of anti-thrombotic drugs, both individually and in combination, that are nowadays on the market. All these drugs have been developed to chemically inhibit platelet activation, with reduced effect on shear-mediated platelet activation. The effect of these agents on shear-induced platelet activation was assessed after exposure to both constant and dynamic shear stress profiles via the hemodynamic shearing device (HSD). Platelets were exposed *in vitro* to 1) low dynamic shear

stress profiles and 2) established high (“hotspot”) stresses, then their degree of activation was characterized. Afterwards, we tested the anti-platelet agents currently on the market, to examine their ability to modulate platelet reactivity after being exposed to shear. In an attempt to extend the scope of the investigation, we thus set up a specific branch of the study to verify the capability of an unconventional chemical agent (DMSO) to inhibit platelet activation after exposure to external mechanical stimuli.

The antiplatelet agents investigated were aspirin, dipyridamole, cilostazol, pentoxifylline, eptifibatide and ticagrelor. The drugs that offered the better behavior in terms of platelet protection were ticagrelor and cilostazol. These two agents have a common effect, which is the inhibition of Adenosine (ADO) or Adenosine Diphosphate (ADP) receptors. This mechanism of action should be further investigated to confirm its eventual ability of really protect platelets from shear stress effects. However, the obtained results suggest that the most common antiplatelet agents, which are normally used in anticoagulation management for patients treated with MCS devices, are only marginally able to protect platelets from the activation effects of physical forces exerted by cardiac assist devices.

New mechanisms of action were studied to solve the burden of heavy antithrombotic therapies that are currently necessary for MCS patients. As previously described, we investigated the effect of DMSO alone as possible platelet membrane fluidity enhancer, thus preventing platelets activation mediated by shear stress. DMSO-treated platelets were exposed to both constant and extremely dynamic shear conditions, the same that were used to assess the ability of common antiplatelet agents to inhibit platelet activation. Moreover, we exposed platelets pre-treated with different concentrations of DMSO to hyper shear using the SCSD. In contrast with the results obtained testing different common drugs, DMSO proved to protect platelets also at the worst shear stress conditions.

Despite the effectiveness of DMSO in reducing shear-mediated platelet activation, its toxic effect on cells precludes any use on humans. Our results should thus be taken as a suggestion that the effect of other organic non-toxic compounds able to penetrate platelet membranes, thus increasing their fluidity, should be investigated. Our analysis indicates that a paradigm shift is required in the development of new antiplatelet drugs for the treatment of shear mediated platelet activation. In particular, the discovery of new agents able to affect the mechanotransduction apparatus may reduce the need of large antithrombotic therapies, offering an effective protection to platelets when exposed to high shear stress conditions.

The *in vitro* experimental campaigns conducted to investigate the effect of both traditional (antiplatelet agents) or alternative (DMSO) pharmacological treatments, on shear-mediated platelet activation, represented the core experimental work of this dissertation. In both these experimental sets, the platelet activity state was assessed using a modified prothrombinase method, the PAS assay, which is well suited for identifying platelets reactivity due to physical forces, such as fluid shear or turbulence. In fact, the PAS assay provides a correspondence between the platelet activity state, caused by previous shear stress exposure, and the consequent thrombin generation, which is detected during the assay. This methodology was always used for research purposes, with reduced attention to its clinical potentialities. For this reason, in this thesis, some additional experimental efforts were spent to tackle a more hybrid approach, investigating actual VADs both *in vitro* and *in vivo* with calves model using the PAS assay. The obtained results permitted to draft preliminary considerations on the utility of PAS assay also for clinical use. In particular, it emerged that this methodology can be successfully used to assess platelet activation. This finding could pave the road for the development of future evolutions of PAS assay mechanism of action, able to spot platelet sensitization in humans, e.g. helping patients to safely control their platelet activation level after the implant of a MCS device.

6.2 Future Directions

In the present work we investigated the effect of several antiplatelet agents on platelets exposed to defined shear stress profiles using two computer-controlled shearing devices. The subsequent platelet activity state was monitored using the PAS assay. This experimental approach resulted to be an extremely reliable methodology, able to precisely assess the different levels of thrombogenicity of cardiac assist devices. The future improvements should aim at extending the application of the present methodology to other devices, such as stent grafts or heat exchangers. In fact, the use of DTE may allow the development of devices able to have a less thrombotic impact on the biological fluids, thus reducing the need for antithrombotic therapies. The use of the above described experimental approach for studying the interaction between antiplatelet drugs and other biomedical devices, apart from VADs, may eventually pave the way for the assessment and development of more effective universal anti-thrombotic treatments.

Moreover, pharmacological research may get real benefit from a deeper investigation of the mechanisms involved in the dynamic aspects of platelet activation using platelet damage accumulation models (Nobili et al., 2008; Sheriff et al., 2013). This combined approach may help in redefining the previous threshold of platelet activation, above which most of the biochemical agents tested appeared not able to protect platelets from shear stress.

Concerning the possibility of better understanding the mechanisms that lie under the mechanotrasduction response to shear-mediated activation, a new pharmacological strategy that does not implicate the use of DMSO may be pursued. Indeed, new connections between the mechanotrasduction apparatus and platelet activation should be investigated to better characterize the interaction between platelets and shear stress and to find new strategies for modulating the membrane biochemical response to physical stimuli.

Finally, the possibility to develop new diagnostic systems able to measure the efficacy of antiplatelet drugs and detect platelet activity state, as the PAS assay does, is at the cutting edge of technology. Indeed, such systems, especially whether implemented into miniaturized devices, might give birth to a new family of portable clinical devices for the monitoring of blood flow conditions, which may significantly improve the quality of life of MCS patients.

Acknowledgements

This study was supported by Regione Lombardia (call per la ricerca indipendente - “Attività di coordinamento e gestione domiciliare di pazienti portatori di dispositivi di assistenza ventricolare (VAD) per il trattamento dell’insufficienza cardiaca terminale: il VAD Coordinator”), Fondazione Cariplo (Grant number 2011-2241) and the National Institute of Biomedical Imaging and Bioengineering Quantum Grant (Award No. 5U01EB012487-00).

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Appendix. Preliminary use of PAS assay with MCS patients' blood

Platelet activity state of three patients that underwent mechanical circulatory support devices implantation (HeartWare HVAD, Thoratec HeartMate II and Syncardia TAH) was monitored via PAS assay for a total duration of three days after the surgery. This pilot study with three patients was conducted to provide preliminary information about the usefulness of PAS assay among the methods used to characterize VAD thrombogenicity.

Experimental protocol

The patients' clinical condition before implant of MCS devices, as well as the protocol used for the study, are described hereinafter.

Patient A was a 59 years old female with past history of diabetes mellitus, pulmonary hypertension and atrial myxoma. She gradually developed mitral regurgitation with incompetent mitral valve leading to extremely dilated left ventricle with an ejection function of 10%. **HeartWare HVAD** was thus chosen for the treatment of the severe heart failure condition and surgery was performed through a robotic implant.

Patient B was a 49 years old male, who arrived to the Sarver Heart Center in Tucson with LVAD thrombosis. He underwent removal of the thrombosed device and placement of a new LVAD (**Thoratec HeartMate II (HM II)**).

Patient C was a 40 year old male with history of dilated cardiomyopathy and biventricular failure, who underwent a Total Artificial Heart (**Syncardia TAH**) implant as a bridge to cardiac transplantation.

15 ml of blood were drawn from patients before the VAD implant, as well as at day 0, 1, 2 and 3 after the surgery. Blood obtained every day was treated as described in the previous chapters and GFP extracted as reported in Paragraph 3.3.2, as well as in the literature (Sheriff J, Bluestein D et al., 2010; Schulz-Heik et al., 2005).

Results

Results obtained, including both platelet activity state and platelets count, are represented in Figure 1.

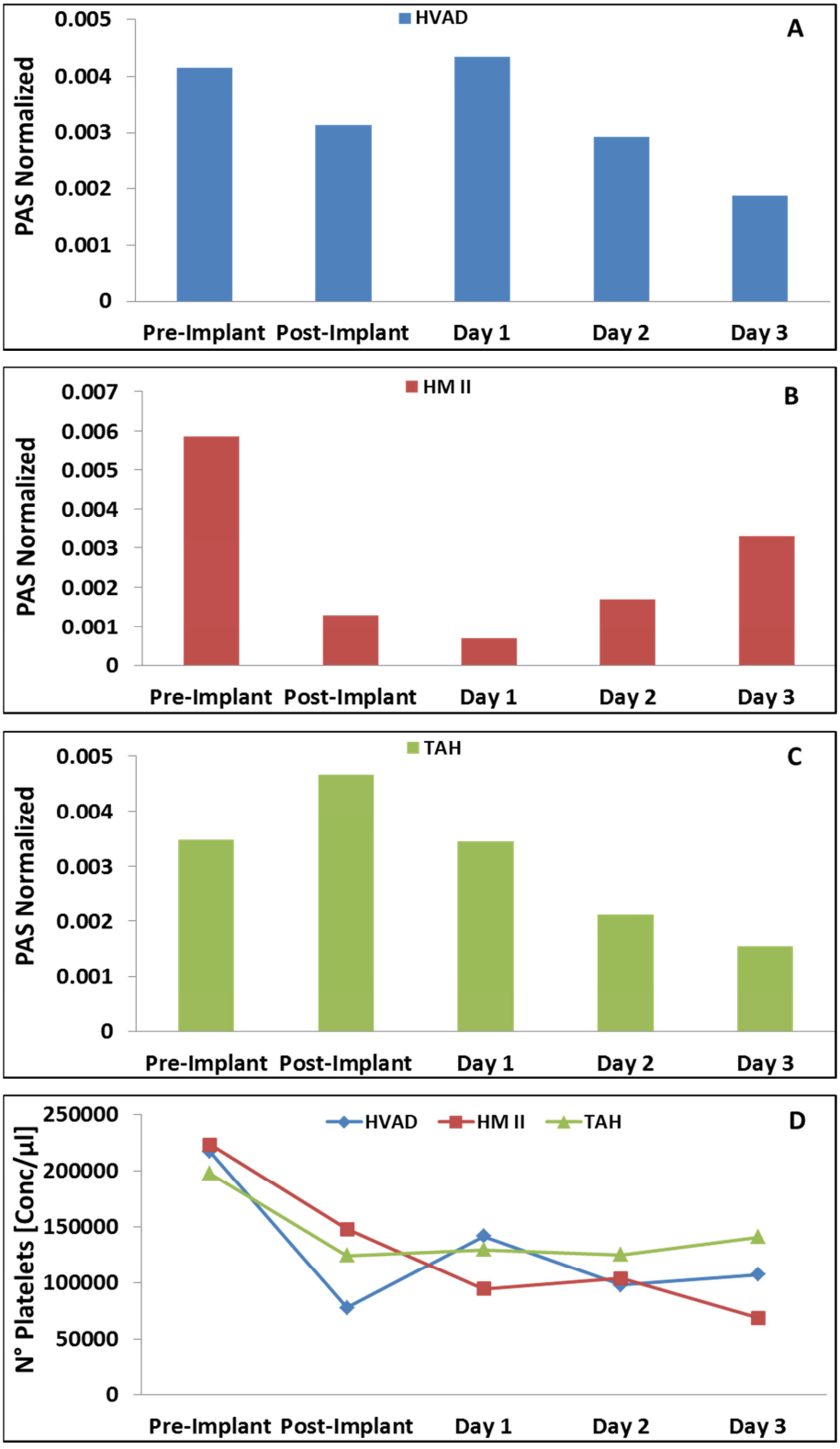

Figure 1. Sensitization of platelets from MCS implanted patients during 3 days after implant of HVAD, HMII and TAH. A-C. PAS of patients monitored up to day 3 after surgery. D. Platelets count.

Data obtained from each patient were evaluated separately. Patient A with HVAD (Figure 1-A) showed a platelet activity state level that remained constant before and after pump implant, with a partial decrease following day 1.

Interesting was the trend obtained with the HMII (Figure 1-B), where platelets appeared highly desensitized after the pump implant, however demonstrating a progressive increase in the following days. The significantly higher pre-implant platelet activity state detected by the PAS assay of the patient treated with HMII may be presumably due to the fact that the patient presented a pump thrombosis that urgently called for substitution with a new VAD.

A different trend was found for the TAH patient (Figure 1-C), where an actual increase in PAS values was detected the day of surgery with a subsequent decrease in the next days. The increase described the day of the implant may be due to the most invasive procedure characterizing a TAH implant, where the whole cardiac structure was substituted with a mechanical device, thus creating an inflammatory response that needed to be controlled with strong medications. This platelet reactivity, phenomenon that was correctly described by the PAS assay, was progressively reduced in the following days.

For what concerns platelet count, a decrease was observed after all the three implants. This phenomenon can be due to the loss of blood during surgery that may have brought to a reduction in platelet count. However, the latter remained stable after day 1, except for the patient with HMII, whose platelet count continue to slightly decrease.

In conclusion, blood from actual MCS patients was tested over a period of 3 days after implant, with the goal to investigate their post-implant platelet sensitization. Among the studies that focus on heart failure treatment, this was the first clinical attempt to connect a pure research method, as PAS assay is, to clinically relevant diagnostic. We believe that data reported need to be supported by a larger pool of cases to further correlate PAS values with clinical data normally used in routinely analysis, as anti-coagulant regimens. This would allow a better evaluation of the behavior of PAS assay as a future method to assess the thrombogenic state of MCS patients' blood. This type of investigation may potentially pave the way for further research studies aimed at realizing diagnostic devices for monitoring the MCS patients' health conditions.