PROTHROMBOTIC EFFECTS OF PREEXISTING THROMBOSIS IN CONTINUOUS FLOW VENTRICULAR ASSIST DEVICES

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Anno Accademico 2015 / 2016
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Abstract

Introduction

Heart failure is a complex and progressive cardiac pathology characterized by structural or functional deficiencies, which determine the inability of the heart to properly eject blood into the circulatory system. In the United States, about 650,000 new cases are diagnosed every year with a 50% of probability of death in the 5 years.

Heart failure can affect the left or the right side of the heart (left or right ventricular heart failure). It can be systolic, meaning that the ventricle does not contract normally, or diastolic, when the ventricle does not relax normally.

At first, this pathological condition can be managed through lifestyle changes or drugs. Nevertheless, as heart failure worsens medical and surgical procedures may be necessary. Heart transplant is considered the best therapy to treat the pathology but, because of the lack of heart donors, this solution is often not achievable. For this reason, in recent decades, ventricular assist devices (VADS) have been developed and used as bridge to transplant (BTT) or as destination therapy (DT).

VADs are battery operated, mechanical pump-type devices surgically implanted. They can be used as a support for the left ventricle (LVAD), the right ventricle (RVAD) or they can assist both ventricles (BiVAD).

Although their efficacy in guarantying survival and a decent quality of life for patients, their use implies risks and complications such as, haemorrhage, infection, device malfunction, acute right ventricular failure and thrombosis.

Thrombosis is considered the most frequent and life threatening risk related to the use of a VAD. It consists in the development of thrombus loads within any component of the device. These clot may cause locally modified fluid dynamics, they may detach from the original site leading to thrombo-embolic events, they may affect platelets causing their activation and the amplification of the thrombotic phenomenon itself. The better understand the effect of thrombosis on platelet activation is the main interest of the present project.
Platelets, the clotting cellular factor, are disc-shaped non-nucleated cells. They take part in the processes which lead to thrombus formation. In fact, chemical and mechanical stimulations of platelets lead to the phenomenon of platelet adhesion, activation and aggregation, which are fundamental for coagulation and thrombus formation. The effect on platelets of pre-existing thrombosis consequent to VAD implantation has not clearly been evaluated. The aim of the present project is to evaluate if VAD-related thrombosis acts as an adjuvant for further platelet activation amplifying, thus, the thrombotic phenomenon itself.

**State of the Art**

Pump thrombosis has been widely diagnosed, observed *in vivo* and reported in literature. It normally develops in an acute way and it is often unpredictable. Therefore, it can be deduced that the phenomenon occurs in a short period of time and this may be related to the fact that a thrombus load may be a powerful platelet activator and, thus, amplify the phenomenon of thrombosis.

To quantify the effect induced by the presence of a thrombus load on platelet activation, the Platelet Activity State (PAS) assay, which has been prevalently used to evaluate shear stress-related platelet activation, has been chosen. It allows the 1:1 correlation between the stimulus induced on platelets by the agonist and the output of the test.

Platelet activation may also be a result of the presence of an interface between platelets and an exogenous material. The contact-related platelet activation is probably the phenomenon occurring in the conditions tested for this project. In fact, platelets interfacing a thrombus load become activated as a result of the contact between the two parts.

**Materials and Methods**

To evaluate the effect of preexisting thrombosis on platelet activation, platelets are interfaced with thrombus loads, both red (mainly consisting in red blood cells) and white (mainly consisting in red platelets). Platelet activation induced by the presence of thrombi is then measured through Platelet Activity State (PAS) assay.
To execute the experiments, blood is drawn from healthy donors, Platelet Rich Plasma (PRP) is obtained by blood centrifugation and Gel Filtrated Platelet (GFP) is obtained by gel-filtration of PRP. Part of whole blood (WB) and PRP is preserved to create red and white thrombi.

Experiments are executed both in static and dynamic conditions to evaluate the effect of convective phenomena, which are very relevant in vivo. Dynamic tests are executed in absence and in presence of a VAD.

Static conditions experiments
Whole blood is inserted at the base of round bottom tubes with volumes equal to 15 µl, 30µl, 60µl and 120µl. The same procedure is followed with PRP added with CaCl₂. After the incubation phase which is necessary to allow clot formation, 4 ml of GFP solution are pipetted on the top of each clot. PAS assay is performed at 1min, 10min and 30min since the beginning of the test.

In addition, few samples are collected and prepare for SEM image acquisition. Both for red and white thrombus, a negative control test is executed by adding 4ml of GFP solution to 120µl of Platelet Buffer. For white thrombus load, a further negative control is executed by adding 4ml of GFP solution to 120µl of PRP not added with CaCl₂ and, therefore, not able to clot.

Dynamic conditions experiments
Dynamic tests are performed to evaluate platelet activation in presence of convective phenomena. To perform this type of tests a hydraulic circuit has been built. It is a closed loop composed of a peristaltic pump, a pump inset and a circuit tube.

Tests are performed at two flow rate conditions, corresponding to two set velocities for the pump, 40rpm and 200rpm. These values are sufficient to generate convective effects but contained shear stresses in order not to obtain shear-related platelet activation.

In order to introduce the presence of a thrombus inside the hydraulic circuit, the clot is prepared on top of a solid metallic frame that is then placed in a fixed location in the circuit.
tube (MESH experiment). The circuit is filled with GFP solution (about 10ml) and PAS assay is executed on samples collected at 1min, 10min and 30min.

For dynamic tests two negative controls are performed by measuring PAS for GFP solution flowing in the circuit at the considered flow rate and for GFP solution at 0rpm.

**Dynamic conditions experiments in presence of a Ventricular Assist Devices**

A preliminary study has been additionally performed by exposing platelets both to the presence of a preformed red or white thrombus load and to shear stress due to the action of the VAD. Due to planning differences between this experimental test and the previous two, this experiment is considered not comparable to the others.

WB and PRP thrombus loads of 120µl of volume, are inserted in a circuit with capacity of about 100ml. The close loop is filled with GFP solution and kept at 37°. The VAD is activated at physiological operating velocity of 9,600rpm and PAS values are measured at 1min, 10min, 30min, 60min and 120min since pump activation.

For both red and white thrombus, two negative control tests are performed by measuring PAS for GFP solution flowing in the circuit at the considered flow rate in absence of the mesh or interfacing a clean one.

**Results**

For all the executed tests, red and white thrombus loads appear to be morphologically different. WB clots appear to be dry, whereas PRP clots appear damp.

**Static conditions experiments**

Activation data for samples interfaced with WB thrombus loads are statistically similar to the values of the negative controls (Fig 0.1). Images acquired by SEM, instead, show activated platelets even at the beginning of the experiment.

PAS results obtained for samples been in contact with PRP clots show a relevant activation for platelets. PAS values for the tested samples are different from both the control tests and proportional to the volume of clot inserted into the tube (Fig. 0.2.).
Fig 0.1.: Platelet Activity State (PAS) due to the presence of WB thrombus load interfacing platelets (Mean and St.Dev). The legend indicates the amounts of clot volume and control solution (Plt buffer) to which 4ml of GFP are added. N experiments = 4 from different donors. *p<0.05.

The activation phenomenon seems to saturate at the beginning of the experiment and, in fact, there are not relevant differences between PAS values for the same samples at different time points.

Fig 0.2.: Platelet Activity State (PAS) due to the presence of PRP thrombus load interfacing platelets (Mean and St.Dev). The legend indicates the amounts of clot volume and control solutions (Plt buffer and PRP not added with CaCl₂) to which 4ml of GFP are added. N experiments = 6 from different donors. *p<0.05. Differences statistically relevant with Control1 are in blue and differences statistically relevant with Control2 are in black.
Dynamic conditions experiments

For a set velocity of 40rpm for the pump, PAS values result to be different from both the controls; tests executed with WB and PRP clots do not show statistical differences at any time point. The phenomenon is quicker at the beginning of the experiment with just a slight increment over time (Fig. 0.3.).

For a set velocity of 200rpm for the pump (Fig 0.4), PAS values result to be generally similar to the one obtained at lower velocity. In fact, the effect on activation of the thrombus is confirmed and no statistical difference is observed between samples tested with WB and PRP clots.

![WB vs PRP 40rpm](image)

Fig 0.3.: Platelet Activity State (PAS) for the dynamic test at 40rpm (Mean and St.Dev). *p<0.05. Differences statistically relevant with Control1 are in blue and differences statistically relevant with Control2 are in black.

The comparison between data obtained for tests at the two velocities shows similar numerical values of platelet activation. Therefore, this result suggests that the introduction of convective effects at low velocities is sufficient to saturate the phenomenon. In fact the increase in convective effects introduced at 200rpm does not lead to further activation for platelets.

Data obtained for red thrombus load through dynamic tests show really different behaviour with respect to static tests. In this case, in fact, WB clots prove to activate platelets and this result is probably related to the introduction of convective effects in dynamic experiments.
Fig 0.4.: Platelet Activity State (PAS) for the dynamic test at 200rpm (Mean and St.Dev). *p<0.05. Statistically relevant differences with Control1 are in blue and statistically relevant differences with Control2 are in black.

**Dynamic conditions experiments in presence of a Ventricular Assist Devices**

The effect of the clot on platelet activation results evident in case of PRP while for experiments executed with WB thrombus loads platelet activation has not been observed (Fig 0.5.).

Fig 0.5.: Platelet Activity State (PAS) for dynamic test with HA5 LVAD (Mean and St.Dev). *p<0.05, **p≤0.1 Statistically relevant differences with Control1 are in blue and statistically relevant differences with Control2 are in black.
Control1 and Control2 seem to be different at 30min and at 60min, suggesting platelet activation over time due to the mesh. The activation given by the presence of the thrombus is confirmed over time, the PAS of the samples been in contact with the PRP mesh differs (with p-value not higher than 0.1) from Control1 and Control2 at various time points, especially after long time since pump activation.

A saturation of the phenomenon has not been observed, probably because of the higher volume of GFP and thus, the higher number of platelets which, in this case, interface the clot.

**Discussions**

**Static conditions experiments**

As observed in the previous section, PAS values for samples been in contact with WB suggested a low activation of platelets, while the correspondent SEM images showed activated cells. WB clots, being dry and attached to the tube surface in static conditions, have probably implied activation just for platelets located at the interface between the GFP solution and the clot or, anyway, very close to the clot. The mixing phase executed right before every collection of the platelet sample (so at 1min, 10 min and 30min) was probably not sufficient to achieve a uniform mixing of platelets in the entire volume contained in the tube.

Results have shown that a PRP thrombus load causes platelets to activate in a way directly proportional to the amount of clot contained in the corresponding round bottom tube. In fact, the higher is the volume of the thrombus, the more are the activated platelets.

The morphological difference between the two typologies of thrombus, WB clots are dry and PRP clots are damp, can be considered as the reason why PAS results for red thrombi are similar to the control results while PAS results for white thrombi are, for high volumes of clot, statistically different from both the considered control samples. In presence of white thrombus load, in fact, GFP solution is probably easily mixed with the clot.

Therefore, there is a high number of platelets which get in contact with the thrombus and platelets get activated more easily in case of white thrombus than with red thrombus.
As a consequence, platelet activation increases as GFP interfaces with a higher amount of clot because the higher is the volume of the clot, the higher is the number of platelets interfacing with it and, thus, activating. Thrombin is considered to be the main agonist for the activation of platelets. In the tests considered for this project, thrombin may still be expressed on the fibrin net of the thrombi or it may be generated by the TF which flows, in a small part, in blood. As platelets get in contact with thrombin, a sequence of events occurs leading to activation of platelets.

**Dynamic conditions experiments**

PAS data for dynamic conditions experiments confirm the phenomenon described for static test results. In fact, in the majority of cases, PAS data for tested samples differ significantly from both the control samples. This difference demonstrates the procoagulant effect of the thrombus on platelets. Results, showing a relevant rate especially at the beginning of the test, can be read as a confirmation of the immediacy of the phenomenon: platelet activation occurs as platelets get in contact with the thrombus. However, unfortunately, it is not possible to explain all the aspects of the phenomenon because of the high number of variables taking part in it and because of the complex, unpredictable and not always repeatable dynamic of the events happening in the circuit as the experiment begins.

Similar evaluations can be made about the comparison between samples tested with WB and PRP clots. The PAS values result to be similar in the two cases at each time point. The relevant difference between the PAS results related to the two type of thrombus described for the static test is not visible for the dynamic one. It is likely that platelets in the dynamic test, being able to flow in the loop, get easily in contact with both PRP and WB thrombus load, even if the latter is dry. However, it is difficult to assert the exact dynamic of what happens to a dry and a damp clot being washed by a platelet solution at various velocities. By comparing results obtained for samples tested at both the velocities, it can be said that the presence of a thrombus load, both red and white, interfacing the platelets implies an increase in platelet activation independently on the velocity at which they are flowing. Therefore, the introduction of convective effects at low velocity is sufficient to saturate the phenomenon and the increment in velocity and in convection does not induce further activation.
Dynamic conditions experiments in presence of a Ventricular Assist Devices

PAS results confirm the overall hypothesis of increase in platelet activation due to the presence of a thrombus load interfacing platelets and they also show, at the end of the test, differences between the two controls. The mesh may cause further platelet activation for two main reasons: fluid dynamic effects due to the presence of the mesh or effects of the mesh material on platelets.

As statistically relevant difference and difference with a significance level not higher than 0.1 are observed between PAS values of these two samples at all the other time points, it can be affirmed that the thrombus load do cause activation for platelets independently on the presence of the stainless steel mesh.

Conclusions

The present project focused on the relevant role of platelets in incrementing thrombosis, once thrombosis is already occurring. The demonstrated activation of cells due to their contact with a thrombus load is important to understand the dynamic of the thrombotic phenomenon. The thesis thus proved that VAD-related thrombosis is an amplifying phenomenon, as it leads to activation of platelets which, in turn, induce the development of thrombosis itself.
Introduzione

L’insufficienza o scompenso cardiaco è una complessa patologia caratterizzata da carenze di tipo strutturale e funzionale che determinano una riduzione della capacità del cuore di eiettare in modo corretto il sangue nel sistema circolatorio. I dati attuali riportano, negli Stati Uniti, più di 650,000 diagnosi di scompenso cardiaco all’anno con una mortalità del 50% entro i 5 anni successivi.

Lo scompenso cardiaco può interessare sia il lato destro sia il lato sinistro del cuore e si definisce sistolico, quando il ventricolo non si contrae in modo fisiologico, e diastolico quando, invece, il ventricolo non si rilassa in modo fisiologico.

Allo stadio iniziale l’insufficienza cardiaca si cura modificando lo stile di vita e con terapia farmacologica adeguata. Nello stadio avanzato della patologia, invece, il ricorso a procedure chirurgiche si rende necessario. Il trapianto di cuore è la migliore terapia per trattare questa patologia ma il ridotto numero di donatori rende questa soluzione non sempre perseguibile. Per questo motivo, negli ultimi decenni, si sono sviluppati dispositivi di assistenza ventricolare (VAD) utilizzati sia come ponte al trapianto, *bridge to transplant*, che come terapia definitiva, *destination therapy*.

I VAD sono pompe meccaniche impiantate chirurgicamente che assistono il cuore in caso di insufficienza cardiaca supportandone l’azione pompante e garantendo il flusso sanguigno in tutto l’organismo. Si parla di LVAD per il cuore sinistro, RVAD per il cuore destro e BiVAD nel caso in cui il cuore abbia bisogno di assistenza bilaterale.

Pur essendo efficaci nel garantire ai pazienti la sopravvivenza e una qualità di vita discreta, il loro utilizzo implica rischi e problematiche come emorragia, infezione, malfunzionamento, ricaduta della patologia sul ventricolo controlaterale e trombosi.

La trombosi è considerata la più frequente e pericolosa delle complicanze relative all’uso di un VAD. Consiste nella formazione di trombi inizialmente adesi alle componenti del dispositivo. Questi coaguli possono sia causare una condizione fluidodinamica alterata, che distaccarsi dal sito originale portando allo sviluppo di fenomeni tromboembolici,

Sommario
che avere un effetto sulle piastrine portando alla loro attivazione e all’amplificazione dello stesso fenomeno trombotico. Lo scopo di questo progetto è comprendere meglio l’effetto della trombosi sull’attivazione piastrinica.

Le piastrine, considerate la componente cellulare determinante per il fenomeno della coagulazione, sono cellule non nucleate dalla forma discoidale. Esse prendono parte ai processi che portano alla formazione di un trombo. Infatti, stimolazioni chimiche o meccaniche delle piastrine suscitano fenomeni di adesione, di attivazione o di aggregazione, fondamentali per la formazione di un coagulo.

L’effetto sulle piastrine della presenza di fenomeni trombotici dovuti all’impianto di un VAD non sono stati finora sufficientemente valutati. Lo scopo del progetto di tesi è proprio quello di valutare se la trombosi indotta dall’impianto di un VAD, oltre a comportare un forte rischio di embolia per il paziente, implichi anche un incremento di attivazione per le piastrine e, quindi, un maggiore rischio di trombosi.

**Stato dell’Arte**

Il fenomeno della trombosi, come conseguenza dell’impianto di un VAD, è stato ampiamente diagnosticato, osservato in vivo e riportato in letteratura. La trombosi ha spesso un decorso acuto ed è difficilmente prevedibile.

Si può quindi dedurre sia che il fenomeno si sviluppi in un breve periodo di tempo sia che un trombo, anche di piccole dimensioni, agisca come potente attivatore di piastrine amplificando, quindi, il fenomeno della trombosi.

Il saggio Platelet Activity State (PAS), utilizzato in letteratura prevalentemente per valutare l’attivazione piastrinica dovuta a shear stress, è stato usato per quantificare l’effetto indotto dalla presenza di un coagulo di sangue sull’attivazione piastrinica. Il saggio PAS permette una correlazione 1:1 tra lo stimolo a cui le piastrine sono sollecitate e l’output del test.

L’attivazione piastrinica può anche essere il risultato della presenza di un’interfaccia tra piastrine e un materiale esogeno. L’attivazione piastrinica dovuta a contatto è probabilmente il meccanismo in atto nei test eseguiti per questo progetto. Infatti, le piastrine che interfacciano un coagulo si attivano per effetto del contatto tra le due parti.
**Materiali e Metodi**

Per valutare l’effetto di una preesistente trombosi sull’attivazione piastrinica, le piastrine sono state messe a contatto con trombi, sia rossi (formati prevalentemente da globuli rossi) che bianchi (formati prevalentemente da piastrine). L’attivazione piastrinica data dal contatto con i coaguli è stata, successivamente, valutata tramite il saggio *Platelet Activity State* (PAS). Per eseguire gli esperimenti, il sangue viene prelevato da donatori volontari, il *Platelet Rich Plasma* (PRP) viene ottenuto tramite centrifugazione e il *Gel Filtrated Platelet* (GFP) viene ottenuto tramite filtrazione del PRP. Sia il sangue intero (WB) che il PRP sono preservati in piccola parte per permettere la formazione dei coaguli rossi e bianchi.

I test sono effettuati in condizioni statiche e in condizioni dinamiche per valutare gli effetti dei fenomeni convettivi fortemente rilevanti *in vivo*. I test dinamici sono effettuati in assenza e in presenza di un dispositivo di assistenza ventricolare.

**Esperimenti in condizione statica**

Volumi crescenti di sangue intero (15 µl, 30µl, 60µl e 120µl) sono posti alla base di uno stesso numero di provette. La stessa procedura è seguita per il PRP con aggiunta di CaCl₂. A seguito della fase di incubazione necessaria a permettere la formazione dei coaguli, 4 ml della soluzione di GFP vengono aggiunti ai coaguli nelle provette. Il PAS test viene effettuato a 1min, 10min e 30min dal contatto tra le due parti. In aggiunta, alcuni campioni sono preparati per l’acquisizione di immagini al microscopio SEM.

Sia per i coaguli di WB che di PRP, viene effettuato un controllo negativo valutando i valori di PAS per GFP aggiunto a 120µl di Platelet Buffer. Per i test con coagulo di PRP viene considerato, in aggiunta, un controllo negativo valutando i valori di PAS per GFP aggiunto a 120µl di PRP al quale non è stato precedentemente aggiunto il CaCl₂, quindi non in grado di coagulare.

**Esperimenti in condizione dinamica**

I test dinamici vengono effettuati per valutare l’attivazione piastrinica in presenza di fenomeni convettivi. Per l’esecuzione di questo tipo di esperimenti è stato necessario
costruire un piccolo circuito idraulico composto da una pompa peristaltica, il corrispondente tubo sotto-pompa e il tubo del circuito.

I test vengono eseguiti a due condizioni di portata, corrispondenti a velocità imposte alla pompa di 40rpm e 200rpm. Questi valori risultano idonei a generare sia effetti convettivi non trascurabili che valori di shear stress non sufficienti ad indurre attivazione piastrinica. La formazione di un coagulo, di volume pari a 120µl, di WB o di PRP avviene su una mesh di acciaio inossidabile successivamente inserita nel circuito (esperimento MESH).

Il circuito viene riempito con la soluzione di GFP (circa 10ml) e il saggio PAS viene effettuato su campione prelevati a 1min, 10min e 30min.

Per i test dinamici vengono considerati due controlli negativi, misurando i valori di PAS per campioni prelevati agli stessi istanti temporali dalla sola soluzione di GFP circolante nel circuito e dalla soluzione di GFP a 0rpm.

Esperimenti in condizione dinamica in presenza di VAD

Uno studio preliminare è stato effettuato esponendo piastrine alla presenza di un coagulo di WB o PRP precedentemente formato su una mesh e di shear stress dovuti alla presenza di un VAD. A causa delle differenze di progetto tra questa e le precedenti campagne sperimentali, questo test non è considerato comparabile con i precedenti due.

Il coagulo di WB o di PRP di volume pari a 120µl viene inserito in un circuito con capacità di circa 100ml. Il circuito viene riempito con la soluzione di GFP e mantenuto a 37°. Il VAD viene avviato alla velocità di 9600rpm, i valori di PAS sono misurati a 1min, 10min, 30min, 60min e 120min dall’attivazione del dispositivo.

Sono stati considerati due controlli negativi misurando i valori di PAS per campioni prelevati agli stessi istanti temporali dalla sola soluzione di GFP circolante nel circuito alla velocità considerata e in assenza della mesh o in presenza di una mesh pulita.

Risultati

Da osservazioni morfologiche si è riscontrato che i coaguli di WB si presentavano secchi mentre i coaguli di PRP si presentavano più umidi.
Esperimenti in condizione statica

I risultati relativi agli esperimenti condotti con coaguli di WB rivelano valori di attivazione piastrinica statisticamente simili ai risultati ottenuti per i controlli negativi (Fig.0.1).

Fig 0.1.: Platelet Activity State (PAS) dovuto alla presenza di un coagulo di WB che interfaccia le piastrine (media e dev. St.). In legenda sono indicati i volumi di trombo e il volume di soluzione (PLt Buffer) utilizzata per il controllo ai quali vengono aggiunti 4ml di GFP. N esperimenti = 4 da diversi donatori. *p<0.05.

Fig 0.2.: Platelet Activity State (PAS) dovuto alla presenza di un coagulo di PRP che interfaccia le piastrine (media e dev. St.). In legenda sono indicati i volumi di trombo e i volumi di soluzione (PLt Buffer) utilizzati per i controlli. A tutti i volumi vengono aggiunti 4ml di GFP. N esperimenti = 6 da diversi donatori. *p<0.05. Le differenze statisticamente significative con il Control1 sono indicate in blu mentre le differenze statisticamente significative con il Control2 sono indicate in nero.
Immagini ottenute al SEM invece mostrano piastrine attivate anche all’inizio dell’esperimento.

I risultati di saggio PAS ottenuti per gli esperimenti con i coaguli di PRP, invece, mostrano un’attivazione piastrinica notevolmente differente da entrambi i controlli e proporzionale alla quantità di coagulo inserito nelle provette (Fig. 0.2.). Il fenomeno di attivazione sembra arrivare a saturazione all’inizio dell’esperimento e, infatti, non si notano variazioni statistiche tra i valori di PAS misurati a diversi istanti di tempo.

Esperimenti in condizione dinamica

Alla velocità di 40rpm imposta alla pompa, i valori di PAS risultano differenti da entrambi i controlli, test effettuati per coaguli di WB e PRP non mostrano differenze e si riscontra una elevata velocità di attivazione all’inizio dell’esperimento con piccoli incrementi nella fase successiva (Fig. 0.3.).

Il fenomeno a velocità di 200rpm per la pompa presenta le stesse dinamiche (Fig. 0.4). I valori di PAS risultano simili a quelli ottenuti a velocità inferiore. Infatti, si conferma l’effetto del trombo sull’attivazione piastrinica e non si osservano differenze tra i campioni testati con coaguli di sangue e di PRP.

Fig 0.3.: Platelet Activity State (PAS) per i test dinamici eseguiti a 40rpm (media e dev. St.). *p<0.05. Le differenze statisticamente significative con il Control1 sono indicate in blu mentre le differenze statisticamente significative con il Control2 sono indicate in nero.
Fig 0.4.: Platelet Activity State (PAS) per i test dinamici eseguiti a 200rpm (media e dev. St.). *$p<0.05$. Le differenze statisticamente significative con il Control1 sono indicate in blu mentre le differenze statisticamente significative con il Control2 sono indicate in nero.

I valori di attivazione ottenuti per le due velocità risultano simili. Si deduce quindi che l’introduzione di effetti convettivi a basse velocità sia sufficiente alla saturazione del fenomeno di attivazione.

Per quanto riguarda i dati ottenuti per i campioni testati con coagulo di sangue, i valori di PAS misurati in condizioni statiche sono notevolmente inferiori rispetto a quelli ottenuti in condizioni dinamiche. In questo caso l’attivazione data dai coaguli di WB si può correlare all’introduzione di effetti convettivi.

**Esperimenti in condizione dinamica in presenza di VAD**

L’effetto del coagulo sull’attivazione piastrinica, mentre risulta evidente per il PRP, non è tale per gli esperimenti eseguiti con i coaguli di WB (Fig 0.5.).

Control1 e Conrol2 risultano diversi a 30 min e 60 min, suggerendo un contributo dato dalla mesh sull’attivazione. Si conferma l’attivazione dovuta alla presenza del trombo.

Non si osserva una saturazione del fenomeno di attivazione probabilmente a causa del maggiore volume di GFP e, quindi, del maggior numero di piastrine che in questo caso interfacciano il coagulo.

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Fig 0.5.: Platelet Activity State (PAS) per i test dinamici eseguiti con HA5 LVAD (media e dev. St.). *p<0.05. **p≤0.1 Le differenze statisticamente significative con il Control1 sono indicate in blu mentre le differenze statisticamente significative con il Control2 sono indicate in nero.

**Discussione**

**Esperimenti in condizione statica**

Come osservato nella sezione precedente, i valori di PAS per campioni testati con WB suggeriscono un basso livello di attivazione piastrinica e sono in contrasto con le immagini SEM che mostrano, invece, piastrine attivate. I coaguli di WB, essendo asciutti, hanno probabilmente causato solo l’attivazione delle piastrine situate in prossimità del coagulo. La fase di miscelazione della provetta, eseguita prima del prelievo di ogni campione si rivela probabilmente non sufficiente ad ottenere un miscelamento uniforme delle piastrine nell’intero volume contenuto in provetta.

Per le piastrine messe a contatto con i coaguli di PRP, invece, i risultati mostrano che l’attivazione è proporzionale al volume di trombo contenuto in provetta.

La differenza morfologica tra i due tipi di trombo (trombi rossi secchi e trombi bianchi umidi) è considerata il motivo per cui i valori di PAS relativi ai coaguli di WB sono simili ai controlli mentre i valori di PAS relativi ai coaguli di PRP sono statisticamente diversi da entrambi i controlli. In questo ultimo caso, infatti, il numero di piastrine a contatto con il trombo è notevolmente maggiore rispetto al caso del trombo di WB.
Conseguentemente, visto che il GFP si miscela meglio con il coagulo, l’attivazione piastrinica è maggiore ed aumenta in relazione al volume di coagulo utilizzato per il test corrispondentemente al maggiore numero di piastrine che interfacciano con esso.

La trombina è considerata il principale agonista che porta all’attivazione piastrinica valutata in questo studio. Essa potrebbe infatti essere ancora espressa sulla rete di fibrina dei coaguli o potrebbe essere generata dal Tissue Factor (TF) il quale è contenuto, seppure in piccola parte, nel flusso sanguigno. La sequenza di eventi che porta all’attivazione delle piastrine si attiva appena le piastrine entrano in contatto con la trombina.

Esperimenti in condizione dinamica

I risultati ottenuti in condizioni dinamiche confermano il verificarsi del fenomeno descritto per gli esperimenti statici. Infatti, nella maggior parte dei casi, i valori di PAS differiscono da entrambi i controlli e ciò dimostra l’effetto procoagulante del trombo sulle piastrine. L’incremento iniziale di attivazione può essere letto come una conferma sull’immediatezza del fenomeno: si suppone infatti che l’attivazione si verifichi appena le piastrine entrano in contatto con il trombo.

In ogni caso, l’alto numero di variabili che incidono sul fenomeno e la complessa, imprevedibile e non sempre ripetibile dinamica degli eventi nel circuito ne rendono difficile la comprensione di tutti gli aspetti.

Simili valutazioni possono anche essere fatte confrontando i dati ottenuti da campioni testati con trombi rossi e bianchi. I valori di PAS risultano simili ad ogni istante di tempo per i due casi. È probabile che le piastrine, in condizioni dinamiche, interagiscano più facilmente con il coagulo di WB, anche se asciutto, e quindi non si osserva, su di esse, la notevole differenza di impatto tra i coaguli di WB e di PRP riportata per gli esperimenti statici. L’esatta dinamica degli eventi in atto durante l’esperimento è però difficile da comprendere.

Il confronto tra i risultati ottenuti alle due velocità suggerisce che l’attivazione da contatto avviene indipendentemente dalla velocità a cui vengono effettuati i test. Quindi l’introduzione di fenomeni convettivi a bassa velocità è sufficiente a saturare il fenomeno e l’incremento di questi effetti non induce un’ulteriore attivazione.
Esperimenti in condizione dinamica in presenza di VAD

I risultati ottenuti confermano il generale effetto sull’attivazione causata dal trombo e mostrano valori di PAS differenti per i due controlli utilizzati. La mesh potrebbe causare un incremento di attivazione per due principali motivi: alterazione della fluidodinamica del flusso in prossimità della mesh o effetti del materiale della mesh sulle piastrine.

D’altra parte però i valori di PAS misurati per i campioni testati risultano statisticamente maggiori rispetto ad entrambi i controlli. L’attivazione delle piastrine si verifica quindi indipendentemente dalla presenza della mesh.

Conclusioni

Il presente progetto pone l’attenzione sul ruolo delle piastrine nell’incrementare ulteriormente il fenomeno della trombosi, quando essa è già in atto. La dimostrazione dell’avvenuta attivazione delle cellule dovuta alla presenza di un trombo è importante per capire la dinamica della trombosi. La tesi quindi prova che la trombosi relativa all’impianto di un VAD è un fenomeno auto amplificante. Esso infatti porta all’attivazione piastrinica che, a sua volta, induce un incremento della stessa trombosi.
Chapter 1 - Introduction

The first chapter of the thesis aims at providing basic information about the area of interest to which the present project can be related.

The first paragraph describes the pathology, heart failure, which pushed us to investigate in this direction. Ventricular Assist Devices (VADs), as the most used therapeutic solution for severe heart failure, and their complication are described in the second chapter.

The thrombosis is considered the most serious risk of VAD implantation, the third chapter provides an overall description of the phenomenon which leads to the formation of a thrombus, haemostasis and, especially coagulation.

The last paragraph describes platelets, the cellular clotting element, and explains their contribution to coagulation through the adhesion, activation and aggregation phenomena.

1.1 Heart Failure

In the United States, about 5.7 million of people (2% of the population) have Heart Failure (HF) and over 900,000 new cases are diagnosed every year. These numbers are increasing over time, as there are more people surviving heart attacks and other acute heart conditions. As people with damaged hearts live longer, they become more susceptible to heart failure [1, 2].

Heart failure is a chronic, progressive condition, defined as the inability of the heart to keep up with the demands of oxygen- and nutrient-rich blood. Specifically, it may indicate heart inefficiency to pump blood (systolic heart failure) or to relax properly to accommodate the blood flow back from the lungs to the heart (diastolic heart failure). Therefore, the heart is not able to provide the proper blood flow to organs such as brain, liver and kidneys [3, 4].

This pathology affects both adults and children. Heart failure can involve the left side, right side or both sides of the heart.

Left-sided or left ventricular (LV) heart failure causes blood congestion in the lungs, which leads to respiratory problems (such as pulmonary edema) and fatigue due to insufficient supply of oxygenated blood to the body.
Right-sided or right ventricular (RV) heart failure is a consequence of pulmonary hypertension and leads to blood stasis in veins and swelling of vessels and organs [5].

Two types of LV heart failure exist (Fig.1.1.):

- **Systolic Heart Failure.** The ventricle does not contract normally and the heart is unable to pump strongly enough to push blood into circulation.

- **Diastolic Heart Failure.** The ventricle does not relax normally and the heart is unable to fill properly with blood.

Heart failure is treated in a specific way depending on the type, the symptoms but also on the class to which it belongs. In fact, the New York Heart Association (NYHA) Functional Classification assigns patients to one of four functional classes, depending on the limitations and symptoms they experience. Whereas, the American College of Cardiology Foundation/ American Heart Association (ACCF/AHA) classification emphasizes evolution and progression of the pathology. These classifications are complementary and meant to be consulted together to define the correct treatment for patients [6].

![Heart failure diagram](image)

**Fig 1.1:** Heart in physiologic condition (in the middle), heart with systolic left heart failure (on the left) and with diastolic left heart failure (on the right)[7].

There are several conditions, such as heart attack, hypertension, coronary heart disease, diabetes, which may lead to heart failure; in general, each situation which damages or overworks the heart muscle can cause heart failure [1].
At first, several physiological mechanisms compensate the effects of heart failure, in order to keep the proper organs perfusion and adequate arterial pressures. The heart starts to pump at a higher frequency to increase the cardiac output. The ventricle chamber dilate with the aim of contracting more strongly (starting from more stretched cardiac fibers). Contracting cells of the heart increase their volume promoting a growth in the muscle mass of the heart. On the systemic level, the body partially diverts blood away from less important tissues and narrows blood vessels to keep a high arterial pressure. These body compensation mechanisms mask the consequences of heart failure just for a limited period, the pathology continues and worsens until these alternative processes no longer work [2, 5].

The signs and symptoms depend upon which side of the heart is failing. The majority of the symptoms are the result of a fluid accumulation in the body due to the increasing volume of the heart chambers. Patients usually experience fatigue, lack of appetite or nausea, swelling especially affecting inferior limbs and veins, frequent urination, increased heartbeat rate, cardiac asthma and breathing problems such as shortness of breath or trouble breathing, which may be a sign of acute pulmonary edema [1, 2].

There is not a specific single test for heart failure. Full evaluation of the patient involves both physical and historical considerations.

Heart failure can not be cured in a definitive way and treatments depend on the type and severity of the pathology. At first, this condition can be managed through lifestyle changes, drugs and ongoing care. Nevertheless, as heart failure worsens, patients may need a medical or surgical procedure.

Implantable heart devices, such as pacemakers and cardioverter defibrillators, are available to improve life duration and quality for patients experiencing asynchronous contraction between right and left side of the heart or irregular heartbeats, both common consequences of heart failure.

About 3.5% of the patients with heart failure in the United States will experience symptoms of advanced heart failure and may be evaluated for heart transplantation, the gold standard and life-saving therapy for severe heart failure [8].

Because of the shortage of organ donors, patients in need of heart transplant go through a careful selection process and, if all the eligibility requirements are met, they will be registered on a waiting list. About 3000 people in the United States are on the waiting list.
for a heart transplantation on any given day and only about 2000 donor hearts are available every year [1].

Since there are important limitations for heart transplantations, in the last decades mechanical circulatory support (MCS) devices, such as ventricular assist devices (VADs), have been the therapeutic way to take over the function of failing heart for a bigger and bigger number of advanced heart failure patients.

This type of MCS devices and the main complications related to their implantation are described in detail in the next paragraph.

1.2 Ventricular Assist Devices (VADs)

Increasing life expectancy has led to a higher number of advanced heart failure patients. Due to the paucity of heart donors, the number of people waiting for heart transplantation and the number of HF patients not eligible for it, because of older age or comorbidity, are continuously raising [9]. Without other optimal solutions, Mechanical Circulatory Support (MCS) devices, such as Ventricular Assist Devices (VADs), have emerged as an important temporary and permanent therapy for advanced heart failure [10].

A VAD is a battery-operated, mechanical pump-type device surgically implanted. Its main function is to unload the failing heart and help maintain the pumping ability of the heart and vital organ perfusion [8].

VADs can be used as a support for the left ventricle (left ventricular assist device or LVAD), the right ventricle (right ventricular assist device or RVAD). Patients diagnosed with failing both left and right heart need a biventricular assist device (BiVAD) (Fig 1.2 a).

LVADs are the most used in clinics, while RVADs are rarely used as an isolated procedure, they are normally necessary after a LVADs or other cardiac surgical procedures. BiVADs are systems of two independent pumps, each of them supporting one ventricle.

There are several components of the VAD (some of which are shown in Fig 1.2.b): a pumping chamber, an inflow cannula, an outflow cannula, a percutaneous driveline, an external controller, which allows the monitoring of the device, and power supply.
Fig 1.2: (a) Schematic representation of Thoratec BiVAD, composed by a RVAD and a LVAD [11], (b) schematic representation of a VAD with all its main components [12].

The pump of the device may be implanted or placed outside the body, in a para-corporeal position. The inflow cannula, attached to the ventricle, and the outflow cannula, attached to the ascending aorta or the main pulmonary artery, allow blood circulation and, especially in recent time, are designed in order to meet anatomical sizes and characteristics of a wide range of patients. Through the cannulas, the device pumps blood from the weakened ventricle to the systemic and/or the pulmonary circulation. Specifically, in case of LVAD, the outflow cannula connects to the aorta, which feeds blood to the entire body, while, in case of RVAD, the outflow cannula connects to the pulmonary artery.

The percutaneous driveline, containing control and power wires, exits the skin, usually on the right side of the abdomen, and connects to a controller and to the power supply.

VADs can be discriminated in pulsatile and continuous flow pumps (Fig. 1.3). Pulsatile pumps have an inflow and an outflow artificial heart valve, needed for unidirectional blood flow, and a pusher plate. Blood is normally ejected 80-100 times per minute, pumping up to 10 litres/min. They are heavy and usually placed outside the body.
In continuous devices, instead, the flow is obtained by using a rotor, which spins up to 15,000 times per minute (with a typical range between 8,000 and 10,000 rpm). The continuous flow has sometimes a possible slight pulsatility, noticeable in a few patients and due to the contractility of the ventricle. This type of pumps, as the number of moving parts is reduced to one (the rotor), is more durable and has a lower probability of complications.

Fig 1.3: Schematic representation of a pulsatile-flow VAD and a continuous-flow VAD with their inner mechanisms [13].
VADs can be powered in two different ways. They can be pneumatically driven, as the majority of pulsatile pumps, or magnetically driven. Pneumatic VADs are cumbersome in volume and intended for a short-term use while the magnetically driven ones are smaller, less limiting and meant for a long-term support [14].

VADs can be used as short-term supports or as long-term supports and they are implanted for three main applications: as Bridge to Recovery, as Bridge to Transplantation and as Destination Therapy.

Bridge to Recovery (BTR) is useful for patients who need just a temporary support (days or weeks). VAD is implanted and, once unloaded, the weakened heart has time to recover and get back to a normal condition. Afterwards, the device is removed and the patient usually comes back to a normal life. BTR strategy may be used for patients with myocardial infarction, acute inflammatory cardiomyopathies or viral illness which constitute <5% of all patients with an implanted VAD [8]. It has been demonstrated that, while the device is implanted, the unloaded ventricle undergoes a complex process of remodelling due to structural and functional improvements of myocardial cells. Alterations at the cellular level lead to a decrease in ventricular size and to the normalization of pressure-volume relation.

Bridge to Transplantation (BTT) represents the group with the highest number of people receiving a VAD. In this case, patients meet all the requirements to receive a heart transplantation but they are too sick to wait for the heart donor. The implanted VAD helps them improve their condition, in particular ventricular function and organ perfusion. This implies a general recovery for the patient and a reduction of the comorbidities, making the patient a better candidate for heart transplantation.

Destination Therapy (DT) strategy is meant for patients who, because of older age or other chronic diseases, are not eligible for heart transplantation. They receive a VAD as an alternative therapy to heart transplant. DT for a VAD has been approved for the first time in 2002 (for HeartMate XVE LVAD, Thoratec Corporation, Pleasanton, CA) and, since then, improvements in quality of life and survival, reduced probability of stroke and device failure, have been demonstrated.
Since the first VAD was made in 1966 (DeBakey VAD, Texas Heart Institute, Houston, TX), the device has been improved and adjusted to meet needs of a wider range of patients. Most important changes by far consist in the transition from pulsatile to continuous flow devices, the reduction in size allowing internal placement of the device and the use of electricity as power supply [9]. Figure 1.4 briefly shows the evolution and the improvements of this device from the first VADs made to newer technologies.

The first generation of VADs mimic the physiological heart function. In fact, they are pulsatile pumps. With respect to medical therapy, these devices improve the quality of life and increase its duration for end-stage HF patients. On the other hand, first generation VADs are heavy and cumbersome in volume with large surgical dissections, increasing the risk of hematomas and infections. Power supply is frequently pneumatic with a noisy system, less comfortable for the patient.

HeartMate XVE (Thoratec Corporation, Pleasanton, CA) is a very used first-generation VAD. Its acceptance for DT in 2002 was not generally accepted because of complications such as sepsis and stroke. This kind of devices are used mainly as BTT therapy.

Second-generation VADs are smaller, lighter and more quite pumps. The reduced dimension makes the surgical procedure safer and makes treatments with VAD an option for a higher number of people. These pumps are continuous devices, It is demonstrated that second generation VADs improve the hemodynamics, the functional capacity and the quality of life of patients. They reduce the probability of reoperation, the rate of rehospitalization, the frequency of complications [9, 10, 13]. These devices are used for long-term support. The HeartMate II (Thoratec Corporation, Pleasanton, CA), the most
successful second generation VAD, was approved by Food and Drug Administration (FDA) for implantation as Bridge to Transplantation in 2008 and as Destination Therapy in 2010. Another example of second-generation VAD is the HeartAssist5 (ReliantHeart Inc., Houston, TX). This device was approved for use in the European Union and, up to date, for use in children in the US.

The main purpose of third generation of VADs is to improve the durability of the device. Drivelines are optimised and pumps work in a centrifugal way and use a magnetically or mechanically suspended rotor. Third generation VADs performance and improvements are still under study.

1.2.1. Ventricular Assist Devices: Risks and Complications

VADs offer a valid life-saving therapy for end-stage heart failure patients but the use of these devices implies a number of different risks. Complications may arise during, right after the surgery or afterwards. Perioperative problems are haemorrhage, right ventricular failure, sepsis, air embolism and kinking of conduits, while late complications include device malfunction, thrombosis, neurologic events and infection.

The risk of a VAD-related bleeding complication is really high both during the surgical procedure and afterwards. VAD implantation is a complex operation, which implies abdominal and chest incisions and extracorporeal circulation. Moreover, anticoagulation therapy, due to the device implantation, increases the risk of early and late bleeding. After the surgery, a high number of patients experience gastrointestinal (GI) bleeding. This phenomenon is associated with different factors. High shear stresses, due to the VAD mode of operation, may cause changing in blood-clotting factors such as von Willebrand factor (vWF), which plays a fundamental role in the coagulation cascade and it is very sensitive to shear stress variations [9].

Infections usually occur between a couple of week to a couple of months after surgery. Both in case of implantable or transcutaneous VADs, the percutaneous driveline is the most common site of infection which can then spread to other part of the system leading to sepsis, bacteraemia and endocarditis. Bacteria involved in the infection are usually common bacteria that live on the skin or bacteria from the gastrointestinal or urinary system [9].
A rare, although possible, risk of VAD implant is the device malfunctions. Since when VADs have been approved for DT, devices were required to be more reliable and last longer (>2 years). The improved durability of newer generations of VADs (continuous flow, easier surgical procedure, low number of movable parts) has been demonstrated. Complications can still arise in any component but these devices have system controllers and displays that, in case of malfunction, provide visual and auditory warnings. Therefore, it can be said that serious worsening of patient conditions due to VAD failure is a rare event [8].

Acute Right Ventricular Failure is, instead, quite common. In fact, this condition occurs in 20-50% of advanced HF patients treated with LVAD. In fact, the right ventricle, used to the performance of a weakened left ventricle, may be too weak to meet the pumping ability of the device, which pumps a higher volume of blood with an increased pumping rate [10].

Thrombosis is considered as the most dangerous and life threatening risk related the a VAD implantation. It consists in the development of thrombus loads within any component of the device. When blood is exposed to a foreign surface, the immune system and platelets may be activated, initiating the coagulation cascade. In addition, surfaces of VADs along with turbulent blood flow increases the risk of exposing blood to variations of shear stress and thrombosis. Anticoagulant and antiplatelet therapies are, therefore, necessary in case of VAD implantation.

Blood clots disrupt blood flow when attached to the device and, more problematically, if they leave the area of the device they can obstruct blood vessels causing a lacking perfusion of important organs. A lack of blood perfusion to the brain, whose vessels are mostly small and thus easy to block, may cause stroke or other celebro-vascular problems, potentially leading to death. Neurologic problems related to VAD thrombosis depend on the characteristics of the device. In fact, design modifications and innovations in materials, such as the use of textured coatings, biological materials, should be useful to reduce the probability of thrombus formation.

It is important to stress that VAD-related thrombosis is usually an acute phenomenon: it occurs in a short time and it is almost impossible to predict it and remedy the problem. Therefore thrombosis often leads to fatal consequences.
The velocity of this event may be linked to the presence of a thrombus load which acts as adjuvant for platelet activation and, thus, amplifies the phenomenon. For these reasons, the aim of the present study is to evaluate the effects of a thrombi on platelets. As thrombosis is a consequence of an altered and undesired haemostasis, next paragraph will describe the phenomenon of haemostasis more in detail.

1.3. Haemostasis

Haemostasis is the phenomenon necessary to keep the integrity of the circulatory system in case of vessel lesions. When the endothelial layer of a vessel is damaged, both circulating platelets and blood coagulation intervene with the final aim of forming a fibrin network, the thrombus, which will block the haemorrhage leading to the healing of the injury. The haemostatic process consists in blood clotting and final dissolution of the thrombus load, once the tissue is repaired [16].

In particular, the haemostatic phenomenon can be schematize in four phases:

- Local vasoconstriction occurs to limit blood perfusion to the damaged area;
- A temporary platelet plug is formed. Platelets are activated by tissue factor or other agonists; they adhere to collagen, which is exposed due to the lesion in the endothelial layer of the vessel, they change their shape, to facilitate plug formation, and they secrete several factors triggering additional platelet activation.
- A thrombus load is formed through the mechanism of blood coagulation. If the clot contains only platelets, it is referred to as white thrombus; whereas it is called red thrombus if it contains also red blood cells.
- The clot is dissolved, by the enzyme plasmin, allowing tissue repair [17].

A relevant role in the haemostatic process is played by the coagulation cascade. Coagulation, in fact, consists in a sequence of several enzymatic reactions with progressive amplification and it leads to the formation of a thrombus load of insoluble fibrin.

Every phase of the coagulation cascade involves an enzyme (an activated clotting factor), a substrate (a clotting factor in its inactive form) and a cofactor, which speeds up the reaction. These components bind on a negatively charged phospholipidic surface, which is mainly formed by activated platelets [2].
Blood coagulation is typically divided into two separate initiation mechanisms, the extrinsic and the intrinsic pathways. All functional factors of the extrinsic pathway are found in extravascular tissue, whereas the factors related to the intrinsic coagulation mechanism are blood borne. Both of them eventually converge to fibrin formation (Fig 1.5).

Blood coagulation follows the extrinsic pathway, or tissue factor pathway, when the endothelium is damaged and blood is exposed to subendothelial matrix, mainly constituted by collagen. Tissue factor (TF), and integral membrane glycoprotein found in
extravascular tissue, initiates the pathway binding to factor VII (FVII) or its activated form FVIIa. TF does not play an enzymatic role in the coagulation cascade but it has two main functions: it acts as cofactor promoting the auto activation of FVII and, most importantly, it raises the enzymatic activity of FVII. The TF:FVIIa complex converts both factor X (FX) and factor IX (FIX) in their activated forms FXa and FIXa, respectively. Both of these activated factors contribute to increase coagulation by feedback activating more FVII in FVIIa.

As FX maintains its bond to the TF:FVIIa complex, once it is activated, hence limiting further activation of FX, the initial amount of FXa formed is poor and not enough to generation a sufficient amount of fibrin. However, this amount of FXa, activates platelets, through PAR receptor, converts factor V (FV) and factor VIII (FVIII), both belonging to the intrinsic pathway, in their activated forms FVa and FVIIIa, respectively. FVIIIa binds with the activated form of FIX, FIXa, constituting the FIXa:FVIIIa complex, which effectively activates a relevant amount of FX. FXa, in high quantity, binds with FVa forming the prothrombinase complex, FXa:FVa. Both the FIXa:FVIIIa complex and the FXa:FVa complex rapidly increase the concentration of thrombin which is now sufficient to lead to the formation of the fibrin network. By the way, these amplification processes, leading to a higher formation of thrombin, are significant just with a low initial exposition to TF.

The activation of FX in FXa is the phase of the coagulation cascade where the extrinsic and the intrinsic pathway converge. [19]

Three plasmatic proteins, initially regulate the intrinsic pathway, or contact activation pathway: factor XII (FXII), prekallikrein (PK) and high molecular weight kininogen (HMWK). The coagulation cascade initiates with the autoactivation of FXII by contact with a surface interface. The active form of FXII, FXIIa, promotes the activation of FXI and PK in FXIa and kallikrein. FXIIa is also a substrate for kallikrein reactions, forming a short reciprocal loop and enabling a rapid activation of the intrinsic pathway.

FXI binds with platelet membrane through GPIb-IX-V receptor and its active form FXIa promotes the activation of FIX. FIXa, in turn, promotes FX activation in FXa, which links intrinsic and extrinsic pathways.
FXa acts on prothrombin but, as both FIXa ans FXa lack of their cofactors FVIII and FVa, it generates just a limited amount of active thrombin. However, the existing thrombin and FXa convert pro-cofactors into active cofactors leading to a rapid formation of thrombin. Both pathways converge at the final steps of the coagulation cascade. Fibrinogen is converted into fibrin molecules, which assemble to form protofibrils and, eventually, fibrin fibers organized in a fibril network. The fibrin plug structure and porosity depend on the environmental factors and dynamic and physiologic conditions. The network is, in the end, stabilized by factor XIII (FXIII), which induces crosslinks between fibrin molecules [19]. The importance of platelets and their role in the coagulation mechanism make it necessary to describe in detail this cellular component of blood and the processes they take part in, which may lead to blood coagulation.

1.4. Platelets

Platelets play an important role in the haemostatic phenomenon. In fact, they form the initial plug, necessary to heal vascular injury at the first phase, and they create a surface, which contains active clotting factors. Platelets are disc-shaped non-nucleated cells with an average diameter of 2.5 µm. They circulate in the circulatory system and are generated by the large megakaryocytes of the bone marrow.

Several glycoproteic receptors are distributed on platelet surface. They facilitate platelet adhesion, activation, aggregation and interaction with blood cells, other platelets and extracellular matrix (Fig 1.6). Platelets activity mainly depends on these receptors, on a contractile cytoskeleton and on cytoplasmatic granules, which, when platelet activation occurs, fuse with the platelet membrane and release their content. There are two main kinds of granules:

- α granules. They express the adhesion molecule P-selectin and they contain proteins involved in the coagulation process, such as fibrinogen, clotting factor FV, von Willebrand Factor (vWF), and protein-based factors involved in wound cicatrisation such as fibronectin, platelet factor 4 and the growth factors Platelet-Derived Growth Factor (PDGF) and the transforming growth beta factor (TGFβ).
- δ granules (dense granules). They contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), Calcium ions, histamine, serotonin and adrenalin. [2]
Following a vascular injury, platelets are in contact with components of subendothelial tissue, such as collagen and vWF. Several reactions take place on the platelet membrane, eventually leading to clot formation.

Platelets start a primary adhesion to the subendothelial matrix, which is mainly constituted by collagen type I or collagen type III. Local flow conditions influence platelets behaviour with high significance. In fact, high blood flow rate implies that platelet concentration is higher next to the wall where also shear stresses are higher, whereas fluid velocity is lower. Bigger blood cells, instead, flow in the central area of the vessel, where velocities are more significant.

When high shear stress conditions occur at the vessel wall, platelets adhesion arises. The bond between glycoprotein Ib/IX/V (GPIb/IX/V) receptor, situated on platelet surface, and collagen is mediated by vWF, a multimeric plasma protein and major ligand for glycoprotein Ib, which rapidly binds to collagen. GPVI receptor binds directly to collagen.
and both GPIb/IX/V and GPVI receptors stimulate platelet activation through outside-in signalling. Another important receptor for platelet adhesion is the integrin α2β1 adhesion receptor, also identified as GPla/IIa, which links to collagen without mediators and it is believed to play an essential role even in platelet spreading on the collagen surface [19, 20].

Platelet aggregation occurs when a primary layer of platelets has already covered the exposed subendothelial matrix. Platelets circulating in the blood flow adhere to the first layer leading to clot formation. The main factor involved in platelet aggregation is integrin αIIbβ3 receptor, also known as GPlib/IIa receptor. It binds to vWF, vitronectin, fibronectin, thrombospondin and, especially, to fibrinogen, with whom it has high affinity after platelet activation occurs. Every single fibrinogen molecule links to two αIIbβ3 receptors, belonging to two different platelets. This way, platelet concentration increases in situ [19, 20].

The initial activation state needs to be propagated to all the platelets, which adhere to the first platelet layer, in order to promote recruiting of cells and increasing in volume of the formed plug. By following autocrine or paracrine pathways of signalling, activated platelets promote platelet activation. Dense granules, or δ granules, secrete ADP and ATP, which activate surrounding platelets through ADP and ATP receptors situated on cells membrane that transmit cell signals by outside-in signalling. In particular, P2X1 is the receptor for ATP, whereas P2Y1 and P2Y12 are receptors for ADP and they are believed to play a role in platelets shape change [21].

Lipid signal molecule thromboxane A2 (TxA2) is synthetized by arachidonic acid metabolism and released from platelets, since the first activation. This molecule is a ligand for TPa receptor and its main function is to promote and help platelet aggregation.

During thrombosis, thrombin enzyme is generated. It has two main functions: it facilitate the fibrin network formation and it activates two platelet receptors called protease activated receptors (PARs) 1 and 4. Thrombin modifies the exposed part of these receptors mediating their activation response. The signal propagates in the intracellular space through outside-in signalling promoted by coupled glycoproteins. PARs bind to the same
molecules (GP) but differ in terms of affinity for thrombin and duration of the intracellular signal [22]. These receptors, which bind to glycoproteins in the intracellular space, are called G-protein coupled receptors (GPCRs). They may be linked to different glycoproteins, leading to different intracellular signalling pathways and different activation responses of platelets.

1.5. Aims of the Project

Mechanical circulatory support (MSC) devices and in particular Ventricular Assist Devices (VADs) have emerged as a vital therapy supporting patients with advanced heart failure. Even if their effectiveness has been confirmed, they implicate serious thrombotic risks such as pump thrombosis, distal thromboembolism and stroke. It has been reported that 70% of VAD patients experience complications in the first year of implantation [23]. Thromboembolic events are mainly due to non-physiologic blood flow in the device. Platelets, the cellular clotting elements of the organism, pass through constricted geometries and may be exposed to quick variations of shear stress, turbulent flow and experience long residence times in pathological flow regions and repeated passages through the device, leading to platelet activation and aggregation. The intent of this project is to better understand the effect of additional contributing factors that can drive VAD thrombosis. In particular, the impact of exogenous thrombus load as an adjuvant to cause platelet activation and, therefore, VAD thrombosis has been examined. The main hypothesis is that an exogenous thrombus, rich in pro-thrombotic mediators, when it is contained in the immediate flow path of platelets, accelerates and amplifies thrombus formation resulting from the presence of the device.

The project has been divided into two main parts. The first experimental step is executed in static conditions with the purpose of examining the effect of preformed white and red thrombus load on platelet activation in vitro. Whereas, in the second part of the project, the impact of white and red clots on platelet activation is studied in dynamic conditions, in recirculating flow loops.

An addition, isolated experimental phase of the project is executed in dynamic conditions by using the HeartAssist5 VAD (ReliantHeart Inc., Houston, TX) willing to have a better understanding on the impact of pre-existing thrombus load on VAD-mediated platelet activation.
Chapter 2 – State of the Art

The present chapter introduces the state of the art about the field of interest. In particular, the first paragraph describes various cases, reported in literature, of ventricular assist devices failure due to pump thrombosis and the consequent findings of thrombus loads in the proximity of the implant. The frequency of these events reveals the interest in studying the effects of the presence of thrombus loads on platelet activation. The second paragraph introduces Platelet Activity State (PAS) assay, which has been used for all the tests executed for this project and allows the evaluation of platelet activation. In literature, PAS has been widely used to evaluate shear stress-related platelet activation and the third paragraph describes a few of these studies. Then, platelet activation due to platelets interfacing an exogenous surface is described.

2.1. VAD-related thrombosis: findings of thrombus loads

Thrombosis is considered the most dangerous complication related to VAD implantation. In fact, although bleeding is more likely to occur after the implant, the consequences of thrombosis can be devastating. Patient would be exposed to the risk of sudden death or to the risk of complex surgical procedure to replace the device and to immediately fix the urgency. Thrombus formation may occur early or late after VAD implantation and it is highly correlated to nonlaminar irregular flow patterns within the device. More in general, factors leading to thrombus formation may be related to the pump, to the patient or to the management of the pharmacological treatment. Since the number of studies and randomized trials about thrombosis occurrence is not sufficient to have a clear understanding of the phenomenon, there is a big possibility of improvement for the knowledge in this area [24].

Pump thrombosis has been widely diagnosed, observed in vivo and reported in literature. In 2012, the study of Park and colleagues [25] aimed at demonstrating the improvements in using continuous flow ventricular assist devices for destination therapy, rather than
pulsatile flow VADs. Authors monitored for two years and compared two groups of patients with same baseline characteristics and treated with HeartMate II (HMII, continuous flow VAD) or HeartMate XVE (pulsatile flow VAD). Although HMII resulted, from comparisons made in terms of survivals, adverse events and quality of life, the device with improved adverse rate events, an equal rate of thrombosis was observed for the two groups. The relative risk ratio of pump thrombosis and pump replacement for pump thrombosis resulted the most relevant in comparison with other diagnosed VAD complications such as, among the others, bleeding, infection, stroke and haemolysis. Thrombosis may lead to potentially fatal cerebrovascular events. Although the immediate perioperative period is associated with a higher incidence of this kind of events, they can also occur throughout the duration of VAD support. 
Lazar et al. [26] reported that a 16% of patients with a VAD had cerebrovascular problems and accidents have increased with a rate of 0.19 per year.

Figure 2.1.: Thrombus in the inflow cannula of a continuous-flow ventricular assist device [27].

Various studies have stated an increase in the observation of this VAD related complication. The spike in incidence of VAD-related thrombosis has been firstly noted
with the HMII in 2011. Since that moment, the problem of pump thrombosis, its implications and relevance as life threatening risk of VAD implantation has been studied in a predominant way. Nevertheless, the reasons of the increase in occurrence of this phenomenon have still not fully clarified.

In 2014, Mehra and colleagues [28] reported occurrence of pump thrombosis, which has increased from 70% to 84%, for HMII as bridge to transplantation, and from 68% to 73% for HMII as destination therapy. They executed a perceptive analysis to describe the factors related to the peak in pump thrombosis rate of recent years. Among these factors, it has been detected the growth of VAD implantations, the implantation in less ill people, the relative increase in DT implantations, the improving survivals and pump characteristics modifications [24].

Pump thrombosis was observed after 3 months from the implantation by Starling and colleagues [29] in 2014. 72 cases of thrombosis were confirmed for 66 patients. Considering the total number of devices with confirmed pump thrombosis over the years, he has clearly confirmed the increase of thrombosis rate over the recent years (from about 1% to about 10% of the implanted VADs).

Najjar and colleagues [30] have investigated the thrombotic phenomenon evaluating also VAD-related thrombosis in case of different surface treatments of the material. They have taken into consideration a list of possible risk factors for pump thrombosis and, through a multivariate analysis, they have studied the occurrence of the phenomenon for each of them. From this analysis, a mean arterial pressure higher than 90 mmHg, an International Normalized Ratio for prothrombin time lower than 2, being on a profile of the Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) between 3 and 7 and an aspirin dose inferior to 81mg are considered predictors of pump thrombosis.

To better understand VAD-related thrombus formation, numerical simulations of the phenomenon have been executed and the overall result confirms the increasing occurrence of pump thrombosis. In 2014, Chiu and colleagues [31] have used advance CFD simulations to predict flow patterns, recirculation zones, stress exposure and stagnant platelet trajectories which promote thrombus formation. In most of the cases, thrombus loads were observed at the flow-straightener and at the rear hub. These areas were proved to be characterized by highly irregular flow patterns within the device. In fact, platelets
form well-defined recirculation zones but platelets trajectories formed also stagnant flow patterns and entrapped circular patterns. Chiu demonstrated the relation between the flow patterns of platelets and the formation of thrombus loads. Pictures in Figure 2.2 show thrombus formation in HeartMate II.

Figure 2.2.: Clinical observation of thrombus formation in HMII. The geometry of HMII flow straightener (A), the velocity flow field along the flow straightener obtained in the study (B) and thrombus formation evidence (C,D,E,F) [31].
Ammar and colleagues [32], in 2012, have used echocardiography to better understand the thrombosis phenomenon. This imaging modality has been proved to be possibly used to assess LVAD implantation and post-LVAD implant cardiac morphology and physiology. Thrombosis prevention is likely to remain a major challenge in the setting of continuous flow VADs and the effort by manufacturers is to optimize device manufacture and implantation through innovation are likely to highlight the need for ongoing analysis of the impact such changes may have on the delicate balance between thrombosis and haemostasis.

Moreover, thrombosis is often an acute complication related to VAD implantation and the event is often unpredictable. Therefore, it can be deduced that the phenomenon occurs in a short period of time and this may be related to the fact that a thrombus load, even with a small size, may be a powerful platelet activator and, thus, amplify the phenomenon of thrombosis.

In recent years, the Platelet Activity State (PAS) assay, which allows to quantify platelet activation induced by an agonist, has been devised. This assay will be described in the following paragraph.

### 2.2. PAS assay

For this project, platelet activation is measured by using a modified prothrombinase method, the PAS assay, developed in 1999 by Bluestein, Jesty and colleagues [33]. The modification of the prothrombinase method, which was ideated by Rosing and Zwaal in the late nineties, allows to obtain a linear quantitative measurement of the initial platelet activation state. This linearity allows, in turn, for a 1:1 correlation between the applied stimulus and thrombin generation, which is then index of induced activation.

Platelets, once activated, expose anionic phospholipids on the outer leaflet of the cell membrane supporting the binding and activation of vitamin K-dependent protein of coagulation (factors VII, IX, X and prothrombin). Furthermore, α-granules of platelets secrete Factor V, concomitantly activated in factor Va. Both anionic phospholipids and factor Va, bind to FXa, which is exposed on the cell membrane, forming the prothrombinase complex (Factor Xa + Va + anionic phospholipid) needed for prothrombin activation and thrombin formation.
The use the kinetics of prothrombin activation, related to platelet activation, as a measure of their procoagulant activity and their activation is not possible through the prothrombinase method because thrombin is an efficient platelet activator. In fact, the presence of thrombin, through a positive feedback mechanism, further activates platelets, leading to higher generation of thrombin. Therefore, the quantity of the initially generated thrombin is unreliable with the prothrombinase method.

Bluestain and Jesty used the same method but with an acetylated prothrombin which does not induce the physiological mechanism of positive feedback. In fact, this prothrombin forms a different kind of thrombin which does no further activate platelets, does not convert fibrinogen in fibrin but can still be detected.

In literature, it has been used a lot to quantify shear stress-related platelet activation. A few of these studies will briefly described in the following paragraph.

### 2.2.1. Shear stress-related platelet activation

The PAS assay has been widely used to assess shear stress-related platelet activation. In 2003, Jesty and colleagues [34] confirmed the increase of platelet activation related to stenosis, a common coronary heart disease. Due to the formation of the atherosclerotic plaque, this pathology causes the exposition of platelets to abnormal flow patterns and high shear stresses for a considerable period of time. Authors studied the dependence of platelet activation on both shear stress and time of exposure. Human platelets were circulated in loops and exposed to various conditions of shear stresses, which were adjusted by varying viscosity, flow rate and time of exposure. The obtained results confirmed that PAS values directly increased with shear stress, duration of the experiment and time of exposure.

The research group composed by Girdhar G., Bluestein D. and Slepian M. [35, 36] used the measurement of platelet activation to assess the efficacy of the Device Thrombogenicity Emulation (DTE), a universal predictive methodology that facilitates the optimization of the thrombogenic performance of any mechanical circulatory support device. In particular, this method integrates numerical simulations with *in vitro* measurements, which resulted by the correlation between platelet activity coagulation markers and the device hemodynamics. Therefore, this method allows an actual optimization of the device in phase of R&D through *in silico* and *in vitro* tests.
The design conditions, optimized with DTE methods, are emulated in a Hemodynamic Shearing Device (HSD). The HSD is programmed to expose platelets to conditions of shear stress which are equal to the ones induced by the device. These conditions of shear stress are valuated through numerical simulations of blood flow throughout the device. Activation was measured through PAS assay and the results confirmed the utility of this methodology in the optimization of thrombogenicity of devices. Although the activation of platelets has been mainly studied when it is related to shear stress, platelets can be activated also as a result of the presence of an interface between platelets and an exogenous material (chemical agonist). The contact-related platelet activation is probably the phenomenon occurring in the conditions tested for this project. In fact, platelets interfacing a thrombus load become activated as a result of the contact between the two parts. The following paragraph will describe a few studies in which contact-related platelet activation is examined.

2.3. Contact-related platelet activation

Besides the mechanical stimulation given by shear stresses, platelets become activated also by the contact with exogenous surfaces. In the present study, in fact, the effects of thrombosis on platelets is studied by interfacing them with a thrombus load and evaluating the activation due to the contact between the two parts. A few studies about the mechanism of contact-related platelet activation are reported in this paragraph.

Sanak and colleagues [37], in 2010 aimed at studying the heamocompatibility of materials. The execution of tests to verify this property are really important for biomedical materials in order to assess the eventuality of adverse interaction between blood and artificial surfaces. Blood was interfaced with several materials and, to evaluate the effects on the cells, various aspects of blood physiology have been considered, such as coagulation, thrombosis, haemolysis and platelet status. Platelet activation was measured by using a flow cytometry analyser and by examining platelet aggregates, platelet activation markers and the concentration of microparticles.
All the tested materials resulted to affect blood by inducing formation of platelet aggregates and platelets-monocytes aggregates, expression of P-selectin and presence of platelet microparticles. These factors are considered indices of platelet activation.

Gemmell and colleagues, instead, focused on studying platelet activation related to the contact between various material and platelets. In fact they attributed the lack of thromboresistant vascular biomaterials especially to platelet activation caused by the contact between the material and platelets,

Authors confirmed platelet activation due to the contact with exogenous materials in a few studies [38, 39, 40]. Fluorescence-activated flow cytometry was used to analyse platelets after they had interfaced various materials, for 1 hour at 37°C, under low shear stress.

For all the tested materials, platelet activation was confirmed by the presence of platelet microparticles, platelet P-selectine expression and the formation of platelet/leukocytes aggregates.

In relation to VAD-related thrombosis and its relevance in the reliability of a device and in relation to the described studies which quantify platelet activation induced by an agonist (chemical or mechanical), in this project we hypothesized that the presence of a preformed thrombus load may act as a powerful agonist for platelets and, thus, cause the degeneration of the phenomenon, leading to compromise both the operation of the device (such as the VAD) and the overall health of patients (with diffused thromboembolic phenomena).

Therefore, the aim of this project is to quantify the effect induced by the presence of a thrombus load on platelet activation. To do so, the PAS assay, which has been prevalently used to evaluate shear stress-related platelet activation, has been chosen.

The PAS assay is used in this case to measure contact-related platelet activation benefiting from its potential to correlate the stimulus induced on platelets by the agonist with the output of the test.
Chapter 3 – Materials and Methods

The present chapter describes the procedure followed for the experiments executed for the study. In particular, the preparation of Platelet-Rich-Plasma (PRP) and Gel-Filtrated-Platelet (GFP) is explained in the first paragraphs. The fourth paragraph describes Platelet Activity State (PAS) assay, which is used to evaluate platelet activation for all the tests. The experimental protocol followed for static and dynamic tests is illustrated in the fifth paragraph, whereas the sixth paragraph explains the protocol for the pilot study executed with LVAD Heart Assist 5. Finally, the adopted procedure of statistical analysis is explained in the last paragraph.

3.1. Introduction

The goal of the project is to better understand the impact of an exogenous thrombus load (red and white) on the pro-thrombotic state, in static and dynamic conditions, by measuring platelet activation with the Platelet Activity State (PAS) Assay.

To execute the experiments, blood is drawn from healthy donors, PRP is obtained by blood centrifugation and GFP is obtained by gel-filtration of PRP. Part of both whole blood and PRP is preserved to create red and white thrombus loads. GFP solution is interfaced with the preformed exogenous red/white clot.

The overall experimental concept is briefly schematized in Figure 3.1.

Experiments are executed both in static and dynamic conditions. The first part of the project consists in interfacing GFP solution with the thrombus load in static conditions, afterwards, in order to study the phenomenon in presence of convective effects, GFP is interfaced with the thrombus load in dynamic condition.

Furthermore, dynamic experimental tests with a HeartAssist5 VAD (ReliantHeart Inc., Houston, TX) are performed to understand the effects of an exogenous thrombus on platelet activation and, consequently, on thrombosis with conditions as close as possible to the in vivo ones. This pilot test is useful to describe the phenomenon at a higher realistic level.
3.2. **Platelet Rich Plasma (PRP) preparation**

About 33ml of human blood is collected from healthy voluntary donors by using a 21G butterfly needle with the Institutional Board Review (IRB) approval at the University of Arizona, in Tucson. Donors, both male and female adults, are asked not to take aspirin or ibuprofen in the 15 preceding days.

A little amount (up to 500µl) of fresh blood is preserved for the experiment with red thrombus loads, the rest is gently mixed in a 50ml Falcon conical centrifuge tube with 10% Anticoagulant Citrate Dextrose Solution A (ACD-A), which blocks the clotting phenomenon during the preparation phase.

In order to separate erythrocytes from platelets and obtain a solution with high platelets concentration, blood is centrifuged at 1500rpm for 13 minutes. The centrifuge (Beckman GS-6R Centrifuge, Beckman Coulter, Miami, FL) divides blood into three different phases depending on the fluid density. In particular, at the bottom of the tube, Red Blood Cells (RBCs) constitute the densest layer; leucocytes and a few platelets constitute an
intermediate white layer called buffy coat and Platelet-Rich Plasma (PRP) constitutes the least dense layer. PRP is collected by using a Pasteur pipette and it is counted through Z2 Particle Beckman Coulter (Beckman Coulter, Miami, FL). The instrument measures the concentration of particles by detecting changes in the impedance of an electrolytic solution when particles pass through a small opening. Before the experiment, the coulter is calibrated to be able to measure the concentration of particles of the same size of human platelets (in a range from 2.03 µm to 4.35 µm).

3.3. Gel Filtered Platelet (GFP) preparation

Before proceeding with gel filtration of PRP and the obtaining of Gel-Filtrated Platelets (GFP), a little amount (up to 500µl) of PRP is preserved for the experiment with white thrombus loads.

In order to obtain GFP, PRP is inserted in a Spectra/Chrome LC column (ID 2.5 cm, volume 4.91ml/cm) containing Sepharose 2B beads (Sigma-Aldrich, St. Louis, MO) with diameter between 60 and 200µm. Beads are equilibrated in (pH 7.4) Platelet Buffer, which does not activate platelets. Platelet Buffer has the following final concentration of solutes: 135 mM NaCl, 10mM HEPES, 5 mM D(+) glucose, 2.7 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM Na₃C₆H₅ and 0.1% bovine serum albumin (BSA).

Gel-filtration of PRP takes advantage of the fact that platelets, which have bigger diameter than plasmatic proteins, proceed faster through the column of beads. Therefore, GFP, constituted only by platelets, is easily collected from the inferior part of the column. Once GFP is collected in a 50ml tube, it is counted through Z2 Particle Beckman Coulter (Beckman Coulter, Miami, FL), as described in the previous paragraph, and it is diluted with Platelet Buffer to obtain a solution with platelet concentration of 20,000 plts/µl. This value of platelet concentration is used to maximize the sensitivity range of the assay and to limit platelet crosstalk and collision [1].

3.4. Platelet Activity State (PAS) Assay

Platelet Activity State (PAS) Assay is used to measure platelet activation for all the experiments executed in this project. PAS Assay allows the measurement of the
procoagulant activity of platelets, and therefore the measure of their activation state, by the quantification of the kinetics of prothrombin activation in presence of platelets.

Prothrombin, once bound to FVa and FXa forms the prothrombinase complex, which converts prothrombin into thrombin. The produced thrombin has a positive feedback response on platelet activation. This makes it difficult to measure the initial generated thrombin.

To avoid this problem and to allow the measurement of initial prothrombinase activity, Jesty and Bluestein in 1999 [33] modified the previous method (the prothrombinase method) by using acetylated prothrombin (AC-FIIa) instead of the physiological prothrombin. This modified prothrombin is activated by the prothrombinase complex but it generates an enzyme that is inactive on fibrinogen and does not further activate platelets (Fig 3.2.).

The removal of the positive feedback action of thrombin, allows to observe the direct relationship between the agonist and thrombin generation, which linearly increases over time.

Fig 3.2: Schematization of the effects Ac-FIIa, used in the PAS assay execution, on the coagulation process. Acetylation stops further platelet activation and fibrin formation [33].
Platelet samples (20,000 plt/µl) collected at different time points for the experimental set-up, are analysed by using the PAS assay. Reagents, which we distinguish in a TUBES solution and in a WELLS solution, are prepared for the experimental procedure.

The TUBES solution contains HEPES-buffered saline with 0.1% bovine serum albumin (HBS:BSA), CaCl$_2$ and Acetylated FIIa (Ac-FIIa), previously added with HBS:BSA and Polyethylene glycol (HBS:BSA-PEG). The WELLS solution contains 1mM Chromazym TH (CH-TH), dissolved in 0.15M NaCl, HBS:BSA + 5mM ethylenediaminetetraacetic acid (EDTA) (HBS:BSA-EDTA). Reagents have to be kept in ice all the time, while CaCl$_2$ may stay at room temperature. 70 µl of tube solution are put in an Eppendorf tube for as many platelet samples will be analysed in the experiment. 150 µl of the WELLS solution are inserted in two wells of a 96-wells microplate for each collected platelet sample.

The spectrophotometer, a 96-well microplate reader (Vmax, Molecular Devices, Sunnyvale, CA), is set to be able to read the sample at an absorbance wavelength of 405nm every 13 seconds for 7 minutes.

25 µl of the platelet sample and 5 µl of FXa are added to the 70µl of TUBES solution, already incubated at 37°C for 10 minutes. After further 10 minutes of incubation at 37°C, necessary to let activated platelets generate thrombin, 10 µl of the incubated tube, containing 5,000plt/µl, are inserted in two different wells. CH-TH binds thrombin and the reaction product colours the solution of yellow. The spectrophotometer analyses the samples, detects the velocity of the reaction and, thus, the quantity of thrombin generated by activated platelets. In fact, the higher is the quantity of generated thrombin, the faster is the reaction.

To obtain platelet activity state, the average of the results of PAS from the two wells, in which each sample is split, is calculated. Platelet activity state of each sample is then normalized by subtraction of a control sample PAS and by dividing this difference by a sonicated sample PAS. The control sample is a GFP sample at 20,000 plts/µl not exposed to agonists and it represents the lowest activation, which may be measured, for a specific sample. The sonicated samples, instead, represents the highest measurable platelet activation result for a specific sample. The platelet sample is sonicated by using a probe-type sonifier (Branson SLPt Sonifier, Branson Ultrasonic Corporation, Danbury, CT) at 50 W for 10 seconds.
3.5. Experimental phase

The main concept of the project is to interface platelets to a thrombus load and to measure platelet activation due to the presence of the thrombus. At first tests were executed in static conditions with the clot as the only agonist, and then in dynamic conditions, adding convective effects to the presence of the thrombus load. Dynamic tests were conducted both in the absence and presence of a commercial VAD.

Both for static and dynamic experiments, white and red clot is formed and it is added to a solution of platelets; PAS assay is executed to measure platelet activation.

The following paragraphs describe in detail the experimental protocol for the executed tests.

3.5.1. Static Condition Experiment

Whole blood is inserted at the base of round bottom tubes with volumes equal to 15µl, 30µl, 60µl and 120µl. The same procedure is followed with PRP. In order to allow the coagulation of PRP, which has been previously mixed with anticoagulant ACD-A, CaCl₂ is added to PRP with a final concentration of 100mM. All the tubes are incubated at 37°C allowing clot formation.

4 ml of GFP solution, containing GFP with 50mM CaCl₂ (final concentration 3mM) and Platelet Buffer, is then pipetted on top of each clot. Since it is impossible to repeat the actual proportionality between the size of a clot attached to an implanted VAD and the volume of platelets flowing in the entire body, it has been considered sufficiently acceptable to dilute the clot under 5% in volumetric ratio (higher volumetric function: 120 µl of clot/4 ml of GFP solution).

At 1 min, 10 min and 30 min since the beginning of the test, the tubes are gently shaken twice, 25 µl of sample are collected from each tube and PAS assay is performed.

In addition, few samples were collected and prepared for SEM image acquisition, in order to allow morphologic assessments of platelets.

For the fixing and the dehydration of sample to be analysed through SEM, the SEM protocol for unfixed platelets is followed. 150µl of platelet sample are added to a glass slide, let to sit and incubated for 15 min. Then, 300µl of 100% Fixator solution (2% V/V of
Glutaraldehyde in Platelet Buffer) is added. After 30 min of incubation, platelets are washed with distilled water, 100% Ethanol and 100% Hexamethyldisilazane (HMDS). Samples are then left to air dry overnight and, once dry, mounted on a support with a double-side carbon tape.

![Fig 3.3.: Experimental set-up for the static test. WB inserted in the tubes in different amounts of volume, Red thrombus load after the test (a, b). PRP inserted in the tubes in different amounts of volume, white thrombus load set up after the test (c, d).](image)

For PAS assay, both for red and for white thrombus, a negative control test is performed by adding 4 ml of GFP solution to 120 µl of Platelet Buffer. For white thrombus load, a further negative control test is executed by adding 4 ml of GFP solution to 120 µl of PRP not added with CaCl₂ and, therefore, not able to clot. Figure 3.3. shows the experimental set-up for red and white thrombus load, after the addiction of WB and PRP volumes and at the end of the test.

The testing conditions and protocols are summarized in Table 3.1.
<table>
<thead>
<tr>
<th>Type of Thrombus/Control</th>
<th>PRP</th>
<th>WB</th>
<th>PRP</th>
<th>WB</th>
<th>PRP</th>
<th>WB</th>
<th>Plt. Buffer (no thrombus)</th>
<th>PRP no CaCl2 (not clotted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. of thrombus/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. of control</td>
<td>15</td>
<td>30</td>
<td>60</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 ml</td>
<td></td>
</tr>
<tr>
<td>Sampling Time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 min, 10 min, 30 min</td>
<td></td>
</tr>
<tr>
<td>Test assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PAS assay and SEM</td>
<td>acquisition</td>
</tr>
</tbody>
</table>

Table 3.1.: Testing conditions for static experiment. The last two columns (in green) are related to the negative controls.

3.5.2. Dynamic Condition Experiment

Tests to evaluate the effects of a thrombus load on platelets in dynamic condition, thus in presence of flow, have been designed and performed. This way, convective phenomena, which are very relevant in vivo, can be take into consideration.

In fact, in patients affected by VAD-related thrombosis, thrombus loads are localized in a few typical sites of the VAD and flowing platelets interface the clots every time they pass through the device.

Indicating with \( t_D \) and \( t_C \) the time of diffusion and the time of convection, respectively, it is possible to calculate an estimate of the relationship between these two parameters:

\[
 t_D \propto \frac{L^2}{D}, \quad t_C \propto \frac{L}{V}.
\]

where \( L \) is the characteristic dimension of VAD, \( D \) is the coefficient of diffusion and \( V \) is the average velocity of blood in the device (\( 10^5 \) cm/s of order of magnitude). Considering thrombin or fibrinogen, whose coefficient of diffusion has an order of magnitude of \( 10^{-7} \)
cm²/s, the relationship between \( t_D \) and \( t_C \) is of \( 10^{-9} \) magnitude. Thus, the time of convection is \( 10^{-9} \) times lower than the time of diffusion and, therefore, convective phenomena result to be very important for blood flowing throughout a VAD.

To perform the dynamic experiments a hydraulic circuit has been built (Fig 3.4.).

![Figure 3.4.: Scheme of the circuit for dynamic experiments.](image)

The circuit is a closed loop composed of a peristaltic pump, a pump inset and a circuit tube. The pump inset and the circuit tube were connected through 2 three ways valves, that were used to fill the system with the sample fluid (GFP) and to collect sample to be analysed over time. The pump is a 520U IP31 Watson Marlow peristaltic pump (Watson-Marlow Fluid Technology, Falmouth, UK). The pump inset is a MasterFlex silicone tubing L/S 15 (Masterflex®, Cole-Parmer, Vernon Hills, IL), 13cm long with 4.8mm (3/16'') of inner diameter (ID) and 2.4mm (1/16'') of wall thickness. The loop tube is a Thermo Scientific 180 PVC tubing (Thermo Fisher Scientific, Waltham, MA), 21cm long with 6.35mm (1/4'') ID, 2.4mm (1/16'') of wall thickness. The ID of the pump inset is considered useful for the range of fluid flows to be tested, whereas the tube circuit has a proper ID to allow the mesh insertion.

Both for WB and PRP thrombus loads, tests are performed at flow rates equal to \( 1.29*10^{-6} \) (40rpm) and \( 5.90*10^{-6} \) (200rpm). These values are sufficient to generate convective effects
but contained shear stresses in order not to obtain shear-related platelet activation. In fact, both the imposed velocities lead to wall shear stresses lower than 1Pa. In particular, 40rpm imply shear stresses of 0.12Pa in the pump inset and of 0.05Pa in the circuit tube, whereas 200rpm lead to shear stresses of 0.59Pa in the pump inset and 0.27Pa in the circuit tube. Tests are performed at two different velocities to study if increased convective effects lead to an increase in platelet activation. In fact, in both cases shear stress in not relevant and differences are only related to the total number of turns and to the convective effects.

In order to introduce the presence of a thrombus inside the hydraulic circuits, the thrombus was prepared on top of a solid metallic frame that was then placed in a fixed location in the circuit tube (MESH experiment).

![Figure 3.5: Pictures (first line) of 3cm² plain mesh (a), mesh covered with WB (b) and with PRP (c). Close-up pictures (second line) taken with optical microscope at 5X mag of the same mesh (d, e, f).](image)

In order to prepare clots on top of a solid substrate, 120µl of either whole blood and PRP (added with CaCl₂ with final concentration of 100mM), were spread onto a 3cm² stainless steel type 316 mesh (wire cloth sheet, Import, Fig 3.5), previously cut in two equal parts. The two rectangular mesh are kept in a closed petri dish and they are incubated at 37°C,
allowing clot formation. Once clots are formed, both mesh are rolled and inserted in the circuit, right downstream and upstream of the pump (Fig 3.6).

Stainless steel is chosen as material for the substrate because of its resistance, elasticity and biocompatibility. In fact, mesh are rolled, inserted in the circuit and they are supposed to keep the initial position during the execution of the experiment. Therefore, the mesh needed to be resistant enough to not break during the insertion phase, to show an elastic behaviour, to keep the grip on the tube wall, and, of course to be hemocompatible and biocompatible to avoid alterations in platelet functionality. The chosen mesh has a porosity of about 0.5 and, by comparison with other mesh initially considered, was evaluated to have a proper porosity to achieve enough uniformity in the substrate covering. To do this, SEM images of the mesh after clot formation were taken. SEM images are acquired by following the same procedure described in paragraph 3.5.1.

The circuit is filled with GFP solution (GFP + CaCl₂ + Platelet Buffer) with a platelet concentration of 20,000plt/µl, the set-up is released from possible air bubbles and the pump is activated. By using an empty syringe and a syringe filled with GFP solution, samples are collected at 1min, 10min and 30min since pump activation. Platelet Activation State (PAS) assay is then performed. Flow rate are, as previously mentioned, equal to 1.29*10⁻⁶ at 40rpm and 5.90 * 10⁻⁶ at 200rpm.
Two negative control tests are performed by measuring PAS for GFP solution flowing in the circuit at the considered flow rate and for GFP solution at 0rpm. The testing conditions and protocols are summarized in Table 3.2.

<table>
<thead>
<tr>
<th>Pump rpm</th>
<th>0</th>
<th>40</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Thrombus</td>
<td>No clot</td>
<td>WB</td>
<td>PRP</td>
</tr>
<tr>
<td>Clot Location</td>
<td>-</td>
<td>MESH</td>
<td>-</td>
</tr>
<tr>
<td>Sampling Time</td>
<td>1 min, 10 min, 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test assay</td>
<td>PAS assay</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.: Testing conditions for static experiment. Columns in green are related to the negative controls.

### 3.5.3. Dynamic Condition Experiment. Effect of thrombus load on VAD-mediated platelet activation

A preliminary experiment in conditions very similar to clinical situation has been additionally performed. Platelet are exposed to PRP and WB thrombus loads and to shear stress due to a relevant flow induced by HeartAssist5 LVAD.

This experiment is not quantitatively comparable with the static and the dynamic tests already described because of differences in the used circuit, in the volumetric ratio of clot over GFP, in velocities and generated shear stresses. However, this pilot test is interesting to describe the phenomenon at a higher realistic level.

The circuit is composed of the VAD, connected to short 0.5” ID Tygon R3603 tubes and a 45” long 0.25” ID Tygon R3603 flow resistor tube (Fig 3.8.). The controller is a MicroMed Clinical Data Acquisition System (CDAS) (MicroMed Cardiovascular Inc, Houston, TX), which monitors the device and provides power.
120µl of whole blood and PRP (added with CaCl₂ with final concentration of 100mM) are spread onto a 3cm² stainless steel type 316 mesh (wire cloth sheet, Import). The mesh is kept in a closed petri dish and it is incubated at 37°C, allowing clot formation. Once clot is formed, the mesh is rolled and inserted in the circuit, right downstream the LVAD, in order to avoid possible movements of the mesh towards the pump. The circuit is filled with GFP solution (GFP + CaCl₂ + Platelet Buffer, concentration 15,000plt/µl) and the set-up is released from possible air bubbles. The whole circuit is incubated at 37°C for the entire duration of the experiment. A temperature sensor is attached to the VAD to monitor the increase of temperature with respect to time during the test. Once VAD temperature is about 37°C, pump is activated at physiological operating velocity, 9,600rpm, corresponding to a cardiac output of about 4l/min.

By using an empty syringe and a syringe filled with GFP solution, platelets samples are collected at 1min, 10min, 30min, 60 min and 120min since pump activation. Platelet Activation State (PAS) assay is then performed at the mentioned time points with concentrations of reagents, which are adjusted to the platelet sample concentration of 15,000 plts/µl.
Both for red and for white thrombus, two negative control tests are performed by measuring PAS for GFP solution flowing in the circuit at the considered flow rate in absence of the mesh or interfacing a clean one.

3.6. Statistical Analysis
Results were processed and evaluated through statistical analysis. First, in order to identify outliers, the normality assumption has been used and values outside the interval \([Q_1-1.5*(Q_3-Q_1), Q_3+1.5*(Q_3-Q_1)]\), where \(Q_i\) is the i-th quartile of the distribution, are not taken into consideration for the PAS results.
Statistical significant differences between different sets of data are determined through a two tails t-test, based on the assumption of equal variance between the two investigated populations of data.
Chapter 4 – Results

4.1. Introduction

The present chapter quantitatively analyses the results obtained for the experiments described in the chapter of Materials and Methods. Platelet Activity State assay is performed to measure platelet activation related to the presence of a preformed red or white thrombus load. The project aims at understanding if an exogenous clot, white or red, interfacing platelets acts as adjuvant for platelet activation.

At first, tests are executed in static conditions with platelets interfacing the clot. At a later time, platelet activation is measured in dynamic conditions, for platelets flowing inside an hydraulic loop and interfacing the thrombus.

In order to replay in a closer way the clinical situation from which the entire project originates, an additional dynamic test is performed by using a HeartAssist5 LVAD. PAS assay is performed for GFP flowing in loop with higher capacity and interfacing a mesh onto which the thrombus has been formed.

The results of these experiments are described in the following paragraphs.

4.2. Morphologic Observations

Thrombus loads were obtained by exposing whole blood and PRP (added with CaCl$_2$ with a final concentration of 100mM) to air and by incubating them at 37°C. After a few hours (normally around 3 hours) of incubation, clots are formed, inserted in the experimental set-up and the test is performed.

For all the executed tests, red and white thrombus loads appear to be morphologically different. In fact, red thrombus appears to be dry and arid. It appears like a solid piece of material. Conversely, white thrombus load, even if clotted, appears to be damp and, with increasing incubation time, there is no change in its consistency.

4.3. Static Condition Experiment

Experiments in static conditions are executed in order to understand, in case of limited convective effects, the effect of an exogenous thrombus on platelet activation and,
consequently, on thrombosis. Both red thrombus load, characterized by predominance of red blood cells (RBCs), and white thrombus load, characterized by predominance of platelets, are formed at the base of round bottom tubes through an incubation phase at 37°C of WB and PRP in different amounts of volume (15µl, 30µl, 60µl and 120µl). Once the clot is formed, 4 ml of GFP are added on the top of the loads. Platelet activation is measured through PAS assay, after a quick phase of shaking, at 1min, 10 min and 30 min since the beginning of the experiment, i.e. the addition of Gel-Filtrated Platelets (GFP) on the tubes containing the clot.

4.3.1. Whole Blood effect

Whole Blood is preserved right after donor blood sample in order to be used to form red thrombus loads. Once clots of 15µl, 30µl, 60µl and 120µl are formed, the experiment begins and Platelet Activity State (PAS) is measured over time for platelets which have interfaced the whole blood (WB) thrombus load in static conditions. In particular PAS assay is performed at 1min, 10 min and 30 min since the beginning of the test. The platelet concentration of GFP solution added to thrombus is 20,000 plt/µl. Activation results provided by the spectrophotometer, a 96-well microplate reader (Vmax, Molecular Devices, Sunnyvale, CA), are normalized with respect to the activation value corresponding to the sonicated sample.

For static tests with WB clot, only one control test is taken into consideration. The evaluated control is the result of platelet activation measured through PAS assay for a sample containing 120µl of Platelet Buffer and 4ml of GFP (20,000 plt/µl). The control result corresponds to the activation of platelets not exposed to any agonists. Even for the control, PAS assay is performed at 1min, 10min and 30min since the beginning of the test.

In order to verify if the obtained data are reproducible and, thus, comparable, platelet concentration is calculated for each category of samples by using the following relation:

\[ C_i \times v_i = C_f \times v_f, \]

where C indicates concentration, v indicates volume and the subscripts i and f indicated the initial and final condition, respectively. The obtained values of concentration are checked to be in the same range.
Considering an average concentration of platelets in whole blood equal to 200,000 plt/µl, platelet concentration is calculated for a solution of 15, 30, 60 and 120µl of WB added with 4 ml of GFP (20,000 plt/µl). The obtained values vary from 20,672 plt/µl (corresponding to the sample with 15µl of WB) to 25,243 plt/µl (corresponding to the sample with 120µl of WB). As these results are calculated as WB was liquid and not clotted, the obtained concentration is the maximum possible concentration for the specific samples and the actual platelet concentration for each sample is lower. Therefore, concentration values belong to the same range of concentration and results can be considered comparable.

The experimental results for GFP exposed to WB clots are reported in Figure 4.1.

![Fig 4.1.: Platelet Activity State (PAS) due to the presence of WB thrombus load interfacing platelets (Mean and St.Dev). The legend indicates the amounts of clot volume and control solution (Plt buffer) to which 4ml of GFP are added. N experiments = 4 from different donors. *p<0.05.](image)

Figure 4.1 shows an overall activation of platelets lower than 20%. The number of executed experiments for each case of different amount of clot is equal to 4. Standard deviations are calculated for each sample and statistical analysis is executed through Student T-TEST with a significance level of 0.05.

The highest activation result (0.12 ± 0.06) is obtained for 120µl of WB at 30 min since the beginning of the test, which corresponds to the highest amount of clot volume and the longest exposure time to the thrombus. Anyway, the statistical analysis has demonstrated that differences are not statistically relevant and none of the PAS results, at any time point, differs from the considered control value. Therefore, even the little increase of values with
respect of time is not relevant as statistically significant differences between any couples of samples has not been obtained.

Platelet concentration is measured by Z2 Particle Beckman Coulter (Beckman Coulter, Miami, FL) for samples tested with 120µl of clotted WB after the conclusion of the experiment (after 30min since the beginning). It resulted to be around 20,000 plt/µl like platelet concentration set for GFP solution.

![SEM images of platelets from samples which have interfaced a WB clot for 1 min (a), 10 min (b) and 30 min (c and d).](image)

Fig 4.2.: SEM images of platelets from samples which have interfaced a WB clot for 1 min (a), 10 min (b) and 30 min (c and d).

SEM analysis is executed for static tests. Figure 4.2 shows platelets collected from samples which have interfaced a WB clot for 1 min, 10 min and 30 min.
Even though PAS results show that WB clots in static conditions seem not to further activate platelets with respect to control samples, platelets showed in SEM images seem to be activated.

4.3.2. Platelet Rich Plasma effect

A little amount of Platelet Rich Plasma (PRP) is preserved before gel filtration of the remaining PRP in order to be used to form white thrombus loads. Since PRP is put in contact with anticoagulant (ACD-A) before blood is centrifuged, a modification of the protocol used to create WB clots is needed in order to let PRP coagulate. Therefore, CaCl$_2$ is added to PRP to obtain a final concentration of 100mM. Once white thrombus loads of 15µl, 30µl, 60µl and 120µl are formed in the tubes, the same procedure followed for WB test is executed. 4ml of GFP (20,000 plt/µl) are added to the round bottom tubes containing the clots and PAS is measured by the 96-well microplate reader at 1min, 10min and 30min since the initial contact between GFP and the loads. Results are normalized as in the previous case.

As described in the Materials and Methods paragraph, two negative control samples are taken into consideration for this test. Control1 is the activation value resulting from 4ml of GFP interfacing 120µl of Platelet Buffer (as in the static test with WB clot). Control2 is the activation value resulting from 4ml of GFP interfacing 120µl of PRP not added with CaCl$_2$, which is not supposed to activate platelets.

As it has been calculated for static tests with WB clots, even for tests with PRP clots platelet concentration is calculated for each category of samples. The number of platelets contained in PRP is averaged from the platelet counts of PRP measured by Z2 Particle Beckman Coulter (Beckman Coulter, Miami, FL) during the experimental execution. PRP platelet concentration results 356,130 plt/µl ± 88,888 plt/µl. Platelet concentration is calculated for a solution of 15, 30, 60 and 120µl of PRP added with 4000µl of GFP (20,000 plt/µl). The obtained values vary from 21,255 plt/µl (corresponding to the sample with 15µl of PRP) to 29,786 plt/µl (corresponding to the sample with 120µl of PRP and to the Control2 sample). Even in this case, these results are calculated as PRP was liquid so, with the exception of the not clotted Control2 sample, the
obtained concentration are higher than the actual ones. Therefore, concentration values belong to the same range of concentration and results can be considered comparable.

Figure 4.3. shows the obtained results. The number of executed experiments is equal to 6 for each category of tested samples. Standard deviation is calculated for each sample and statistical analysis is executed, with a significance level of 0.05, to identify statistically significant differences between samples and both controls.

Control1 and Control2 result similar at every time point, meaning, as supposed, that the presence of not clotted PRP does not drive platelet activation.

Samples tested with 60µl and 120µl of clot differ from Control1 samples at every time point. Samples tested with 120µl of clot result to be different from Control2 PAS at every time points whereas, samples tested with 60µl of clot differ from Control2 in 2 cases out of 3.

The Student T-Test is also used to compare PAS results of samples tested with different amounts of clot. In particular, statistical analysis confirms the differences between PAS for samples tested with 15µl of PRP clot and all the other samples at every time point and the differences between PAS for samples tested with 30µl of clot and 120µl of clot at every
time point. It can be said that samples show that platelet activation increases in a directly proportional way with respect to the amount of clots contained in the tube. The increase of activation over time is instead negligible. Figure 4.4. shows platelet activation state over time.

![Platelet Activity State (PAS) vs Time](image)

**Fig 4.4.:** Representation over time of Platelet Activity State (PAS) due to the presence of PRP thrombus load interfacing platelets (Mean and St.Dev). N experiments = 6 from different donors.

Activation values are stable after the first minute of the experiment and the presence of a plateau for the curves represented in Figure 4.4 is evident. A Student T-TEST with coupled populations of values is executed to determine if differences among PAS of the same group of samples at various time points are statistically relevant. Differences among platelet activation for the same samples but at different time points result negligible for the nearly totality of the comparisons. Differences of PAS values for the same sample interfaced with PRP clots at different time points are calculated and showed in Table 4.1. At 1 min the increase of platelet activation, especially for samples been in contact with higher volume of thrombus, is relevant and PAS values rest mostly stable over time.

It can be also noticeable that samples tested with 15µl of PRP clot seem to activate less than samples of Control2, but in 2 cases out of 3 activation values have not been resulted statistically different.
Table 4.1. Differences in PAS values for the same sample interfaced with PRP clots at different time points. \( \Delta_{0-1} \), \( \Delta_{1-10} \) and \( \Delta_{10-30} \) indicate PAS differences between the value at 1min and the initial one, between the value at 10min and the one at 1min and between the value at 30min and the one at 10min, respectively.

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>( \Delta_{0-1} )</th>
<th>( \Delta_{1-10} )</th>
<th>( \Delta_{10-30} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>120µl</td>
<td>0.728452</td>
<td>-0.00038</td>
<td>-0.06827</td>
</tr>
<tr>
<td>60µl</td>
<td>0.475041</td>
<td>0.0308</td>
<td>-0.00691</td>
</tr>
<tr>
<td>30µl</td>
<td>0.173503</td>
<td>0.041947</td>
<td>0.039229</td>
</tr>
<tr>
<td>15µl</td>
<td>0.023281</td>
<td>-0.00273</td>
<td>0.022119</td>
</tr>
</tbody>
</table>

Fig 4.5.: Formation of further thrombus for 120µl PRP sample. Thrombus inside the tube (a), thrombus taken outside the tube (b).

Moreover, for samples been in contact with 120µl of PRP, the formation of further thrombus originating from the initial exogenous clot is visible (Fig. 4.5.).

Even in this case, platelet concentration is calculated by Z2 Particle Beckman Coulter after 30min since the beginning of the experiment. The concentration value seems to confirm the supposed formation of further clot. In fact, platelet concentration results to be around 810 ± 88 plt/µl, that is much less than the initial concentration.
Even in this case SEM analysis is executed. Figure 4.6 shows platelets collected from samples which have interfaced a PRP clot for 1 min, 10 min and 30 min. Platelets appearance confirm the PAS results.

Fig 4.6.: SEM images of platelets from samples which have interfaced a PRP clot for 1 min (a), 10 min (b) and 30 min (c).

4.4. Dynamic Condition Experiment - MESH Experiment

Experiments in dynamic conditions are executed in order to study the consequences of the presence of an exogenous thrombus on platelet activation and, consequently, on thrombosis, in case of increased convective effects.
Both red and white thrombus loads are formed by spreading 120µl of WB or PRP (added with CaCl₂ with final concentration of 100mM) on a 3cm² stainless steel mesh previously cut in two equal pieces. Mesh are incubated at 37°C until the clotting phenomenon occurs.

![SEM images](image)

Figure 4.7.: SEM images of WB clot on the stainless steel mesh before (a) and after the experiment (b), PRP clot on the mesh before (c) and after the experiment (d). It is visible the thrombus uniformity for WB which is instead less achieved for PRP clots.

Once the clot is formed, the two rectangular mesh are inserted in the built circuit. The latter is filled with GFP solution (20,000plt/µl) and the pump is activated to begin the test. Platelet activation is measured through PAS assay at 1min, 10 min and 30 min since the activation of the pump.
The experiment is executed with imposed 40rpm and 200rpm of velocity for the pump. The increase of pump speed simply aims at adding further convective effects to platelets flowing in the loop.

Scanning electron microscope (SEM) has been used to obtain image captures of the mesh after the clot formation and after test execution. WB thrombus loads show uniform covering of the mesh, while for PRP samples the covering is not total (Fig 4.7.). This difference may be due to the fact that PRP is damp and, therefore, it tends to adhere more to the wires leaving less material in proximity of the porosity. Alternatively, it may also be that PRP clots, not being dry, get washed and partially removed during the fixing and dehydrating phase, which is required for SEM images acquisition.

Figure 4.8. is a SEM image showing an interesting detail of a mesh with WB thrombus load after the experiment.

For the dynamic test executed at 40rpm, two negative control samples are taken into consideration. Control1 is the activation value resulting from GFP staying inside the circuit at 0rpm and not interfacing any mesh. Control2 is the activation value resulting from GFP flowing in the circuit at 40rpm without interfacing any mesh.
Shear stresses are meant to be really low in order not to imply a shear stress-related platelet activation. They are calculated at the inner wall of the circuit for a set velocity of 40rpm and they result to be not higher than 1 dyne/cm² (in particular they are equal to 1.2dynes/cm² in the pump inset and 0.5 dyne/cm² in the PVC circuit tube). The velocity of platelets is 0.04 m/s in the circuit tube and 0.072 m/s in the pump inset and they take 7 seconds to complete one turn.

Figure 4.9. shows the obtained results.

Statistical analysis is executed through Student T-TEST with a significance level of 0.05. Control1 and Control2 result similar at each time point. Therefore, pump itself does not imply further activation for platelets. Statistically relevant differences between samples tested for the experiment and samples of both controls indicate the supposed activation of platelets due to the presence of white and red thrombus loads.

![Graph showing Platelet Activity State (PAS) for the dynamic test at 40rpm (Mean and St.Dev). *p<0.05. Statistically relevant differences with Control1 in blue and statistically relevant differences with Control2 in black.](image)

Figure 4.10. shows the same data over time. Both for red and white thrombus loads, the phenomenon prevalently occurs in the first minute of the experiment. Afterwards platelet activation continues to increase but with a slight slope and without statistically significant differences between samples of the same type collected over time.
The same test is executed also at increased velocity (200rpm) to add further convective effects to the set up. In this case, the highest shear stress result to be around 6 dynes/cm$^2$ (in particular shear stresses are equal to 5.9 dynes/cm$^2$ in the pump inset and 2.7 dynes/cm$^2$ in the PVC circuit tube). The velocity of platelets flowing in the built circuit is 0.19 m/s in the circuit tube and 0.33 m/s in the pump inset and they take 1.50 seconds to complete one turn.

Statistically relevant differences with Control1 are in blue and statistically relevant differences with Control2 are in black.
Figure 4.11 and Figure 4.12 show the results of the test. Even at higher velocity, statistical analysis (with a significance level of 0.05) confirms that there is not difference between values obtained for Control1 and Control2 at each time point. So the pump does not affect platelet activation even with increased convective effects.

It results, especially at 10 min and 30 min since the beginning of the experiment, that the presence of white or red thrombus implies further activation for platelets. The phenomenon of platelet activation occurs at the beginning of the experiment in the range of time included between 1 and 10 min since the pump activation.

No statistically relevant difference between WB and PRP samples has been observed through T TEST at any time point.

The comparison between data obtained for tests at 40rpm and data obtained for tests at 200rpm shows similar numerical values of platelet activation. Therefore, this result suggests that the introduction of convective effects at low velocities is sufficient to saturate the phenomenon. In fact the increase in convective effects introduced at 200rpm does not lead to further activation for platelets.

Data obtained for red thrombus load through dynamic tests show really different behaviour with respect to static tests. In this case, in fact, WB clots prove to activate platelets and this result is probably related to the introduction of convective effects in dynamic experiments.
4.5. Pilot Study: Dynamic Condition Experiment with HA5 LVAD

In addition to the described testes executed in dynamic situation, a pilot study in conditions very similar to the clinical one has been performed. In this case, HeartAssist5 LVAD is the pump used to make platelets circulate. They flow in a bigger flow loop into which a 3cm² mesh, used as support for the formation on red and white thrombus loads, is inserted. The aim is to study the effect of an exogenous thrombus on platelet activation and, consequently, on thrombosis with conditions as close as possible to the *in vivo* ones.

120µl of PRP (added with CaCl₂ with final concentration of 100mM) and 120µl of WB are positioned on the square mesh, trying to obtain a good uniformity in the coverage of both sides of the mesh. Mesh are positioned in petri dish and incubated at 37°C. Once the clot is formed, the clotted mesh is inserted in the loop and the circuit is filled with GFP solution (at 15,000 plt/µl) and incubated. LVAD is activated with a set velocity for the pump of 9,600rpm which corresponds to about 4 l/min of flow rate. The set flow rate is similar to the one flowing through an implanted device as 5l/min is the flow rate through the body but approximately only the 70% of this amount flows even through the VAD. The averaged velocity of platelets inside the loop is about 0.56 m/s and they take around 1.79 s to complete one turn.

Platelet activation is measured through PAS assay at 1min, 10 min, 30 min, 60 min and 120 min since the activation of the LVAD.

This experiment is not quantitatively comparable with the static and the dynamic tests already described because of differences in the circuit used, in the volumetric ratio of clot over GFP, in velocities and generated shear stresses. However, this pilot test is interesting to describe the phenomenon at a higher realistic level.

Figure 4.13. shows the obtained results.

As described in the Materials and Methods paragraph, two negative control samples are taken into consideration for the test. Control1 is the activation value resulting from platelets flowing in the loop without interfacing any mesh. Control2 is the activation value resulting from platelets flowing in the loop and interfacing a clean mesh.
Fig 4.13.: Platelet Activity State (PAS) for dynamic test with HA5 LVAD (Mean and St.Dev). *p<0.05. **p≤0.1 Statistically relevant differences with Control1 are in blue and statistically relevant differences with Control2 are in black.

Standard deviations are calculated for each sample and statistical analysis is executed through Student T-TEST with a significance level of 0.05. The graph highlights with two stars the cases for which the p-value is not higher than 0.1. Considering the variability of the data, these differences can be considered as almost significant.

Control1 and Control2 seem to be different at 30min and at 60min, suggesting platelet activation over time due to the mesh, whereas there is no statistically relevant difference between the two at 120 min. The activation given by the presence of the thrombus is confirmed over time, the PAS of the samples been in contact with the PRP mesh differs (with p-value not higher than 0.1) from Control1 and Control2 at various time points, especially after long time since pump activation. WB mesh seems not to activate platelets. Figure 4.14. shows platelet activation results over time. In general, an effect of thrombus on platelet activation is evident for PRP mesh and not for WB mesh.
In contrast to the static and dynamic test described in the previous paragraphs, for dynamic test with LVAD there is not a phase of plateau. This may be due to the higher number of platelets interfacing the mesh in this experiment, implying a longer time to get a high percentage of platelet in contact with the thrombus and, thus, activated. Platelet count, for this test, is measured after sample collection at each time point. Platelet count result to maintain its original value over time, probably due to the higher volume of GFP solution used to fill the circuit.
Temperature is measured by attaching a temperature sensor at the lateral body of the incubated LVAD. Figure 4.15. shows temperature trends for all the considered samples. Temperature increases quite quickly at the beginning of the experiment whereas, in the second hour of the test, curves show a phase of plateau and temperature tends to be stable. The total increase of temperature is about 10°C.
Chapter 5 – Discussion

5.1. Introduction

The present study aims at assessing whether the presence of an exogenous thrombus, both platelet rich plasma-white and whole blood-red, interfacing platelets acts as an adjuvant for further platelet activation.

Thrombosis is one of the most dangerous and the most common cause of failure for Ventricular Assist Devices (VADs). It is the major pathology derived from haemostasis and it can be due to an endothelial injury, to hypercoagulability and to abnormalities in blood flow. These three elements, described by Virchow and forming the Virchow’s triad, may arise both at the same time and separately. In case of VAD, the physiological blood flow is modified in a relevant way: blood flows in channels which are stiffer and less compliant than blood vessels; blood velocity may vary quickly depending on its pathway and on the site shape; therefore, blood flow may be not everywhere lamellar leading to the presence of vortex or stasis areas.

The multiple findings of thrombus loads attached to the walls of explanted VAD pushed us to better understand the thrombotic risk of VAD implantation and whether or not the presence of thrombi interfacing platelets may lead to further complications.

The thrombus load is basically a net of activated platelets and fibrin which entraps flowing red blood cells. Platelets are considered the cellular clotting elements of the organism. Their activation, adhesion to the site and aggregation are fundamental for the coagulation cascade and the clotting phenomenon.

Platelet activation has been widely studied when it is shear-related but not much has been described about the effect on activation for platelets been in contact with a thrombus.

Therefore, the present project aims at assessing and describing the possible thrombus-related platelet activation. The study has been divided in two main phases: a static test and a dynamic test. Lastly, an additional test, not correlated with the previous two, has been performed, by using a VAD, in conditions very similar to the clinical one.
5.2. Static Condition Experiment

For the static test, experiments were executed by forming both red clots and white clots. Both of these two types may form in case of complications for a heart failure patient treated with VAD. In the Results chapter, morphological differences between red and white thrombi load have been stresses. In fact, the former appeared to be very dry, while the latter was damp and more moist.

Whole blood thrombi consist of fibrin, a high content of red blood cells (RBCs) and a low percentage of platelets. They form with low flow rate condition and are characterized by a weak adherence to a substrate and thus a high risk of embolization. On the other hand, PRP thrombi consist of fibrin, a low content of RBCs and a high percentage of platelets. They likely form with high flow rate condition and are tightly adherent, possibly leading to obturation and peripheral ischemia. Therefore, depending on the type of flow existing in a local area of a VAD component, there may be formation of red or white loads.

Platelet Activity State results for static tests with WB clots have been showed in the previous chapter. Considering Figure 4.1., red thrombus loads seem not to activate platelets in static conditions: PAS results to be lower than 20% for each tested volume of clot and values result similar to the chosen control. As control test, PAS has been executed for a sample containing Platelet Buffer and the GFP solution. The obtained PAS results, by the way, are in contrast with Figure 4.2. showing activated platelets captured by SEM at 1min, 10 min and 30 since the beginning of the test.

As the fixing and dehydrating procedure of SEM analysis entraps with higher efficacy activated platelets than not activated platelets, it can be said that the majority of platelets which had been activated ended up in the SEM images. Therefore, the similarity of PAS results for the tested sample to the results for the control sample does not mean that red thrombus loads do not imply activation for platelets. Instead, it can be affirmed that, WB clots, being dry and attached to the tube surface in static conditions, have implied activation for platelets located at the interface between the GFP solution and the clot or, anyway, very close to the clot. The mixing phase executed right before every collection of the platelet sample (so at 1min, 10 min and 30min) was
probably not sufficient to achieve a uniform mixing of platelets in the entire volume contained in the tube.

For static experiments executed with white thrombus loads, two controls have been considered. The first control (Platelet Buffer and GFP solution) is useful to demonstrate that platelets contained in the GFP solution are not originally activated. The second control (120µl of PRP not added with CaCl₂ and GFP solution) is useful to demonstrate that PRP itself, not coagulated, has no influence on platelet activation.

PAS results, especially for higher volume of clot, differ in a statistically significant way from both the control values. Results have shown (Fig 4.3.) that a PRP thrombus load causes platelets to activate in a way directly proportional to the amount of clot contained in the corresponding round bottom tube. In fact, the higher is the volume of the thrombus, the more are the activated platelets.

The fact that there are no relevant differences between the tested samples and Control2 samples also demonstrates that the activation measured by the spectrophotometer (Vmax, Molecular Devices, Sunnyvale, CA) is actually the activation of platelets and there are no errors in the measurements due to the colour of the sample.

The morphological difference between the two typologies of thrombus, WB clots are dry and PRP clots are damp, can be considered as the reason why PAS results for red thrombi are low (meaning similar to PAS values for control samples) while PAS results for white thrombi are, for high volumes of clot, statistically different from both the considered control samples. In fact, the majority of platelets collected from the sample containing the WB clot have not probably been in contact with the clot itself because the phenomenon of activation happens at the interface between the GFP solution and the clot and, as the clot is dry and stable at the base of the round bottom tubes and the collection of platelets was random from an area at the middle of the tube, it is likely that the collected platelets have never been in proximity of that interface.

In case of PAS executed in presence of white thrombus load, instead, as clotted PRP is not dry, GFP solution is easily mixed with the clot. Therefore, there is a high number of platelets which get in contact with the thrombus and, although the mixing phase for the
static test in both the conditions is equivalent, platelets touch and, thus, get activated more easily in case of white thrombus than with red thrombus.

As a consequence, platelet activation increases as GFP interfaces with a higher amount of clot because the higher is the volume of the clot, the higher is the number of platelets interfacing with it, being in contact with it and, thus, activating.

Interestingly, as shown in Figure 4.5, for samples tested with PRP clots and especially with high volume of clot, the formation of further thrombus originating from the initial exogenous clot is visible. Platelet count measured at the end of the experiment (at 30min since the beginning) confirms the reduction of number of platelets which have been entrapped in the formation of new thrombus.

For these samples, platelet images taken through SEM analysis, show activated platelets at every time point. Anyway, platelets look different from the ones got in contact with WB clots. For samples tested with red clots, platelets are easily discernable, they have very long pseudopods which connect them one another. For samples tested with white clots, instead, platelets appear activated but not easily identifiable. They are connected one to another but pseudopods are rarely visible. Platelets aggregate and seem to have a different, rougher surface.

An hypothetic explanation to their different appearance may be that, as for PRP loads there is formation of further thrombus, the collected platelets have actually some part of thrombus attached to them.

In the Result chapter it was also assessed through Figure 4.4, the immediacy of the activation phenomenon. In fact, the final activation obtained for platelets at 30 min of the test is very similar to the activation measured at 1 min since the beginning. Basically, platelets are mixed with the clot as they get in contact with it and activation occurs immediately after the contact between the two parts. After the first minute of test, there is the saturation of the phenomenon.

Platelet activation may occur by following two independent pathways being initiated by the exposure of subendothelial collagen or by the presence of thrombin, which is probably attached to the clots or generated by tissue factor (TF). In the second case, platelet activation does not require a disruption of the endothelium and it is independent of von
Willebrand factor. Moreover, as tissue factor results to be present not only in subendothelial tissues and in leucocytes but also in platelets and in flowing blood, it may be assumed that platelet activation observed in the present study is due to the mechanism described in the second pathway [41].

Red and white thrombus loads, differ a lot for cellular components but they have similar protein-based and fibrous components. These components, especially the fibrin net which may still express thrombin, act as agonists for platelet activation.

Thrombin is also generated by the TF. In fact, TF binds to factor VIIa and the formed complex of the two factors activates factor IX, thus initiating a proteolytic cascade that generates thrombin [41].

In any case, thrombin is considered to be the main agonist for the activation of platelets. It activates platelets at extremely low concentrations. PAR1 is the protease-activated receptor exposed on the cell surface. Thrombin bind to PAR1 inducing platelet activation. Once the cell is activated, it releases adenosine diphosphate (ADP), serotonin, ephinephrine and thromboxane A2 amplifying, this way, the activation mechanism. Besides inducing platelet procoagulant activity, thrombin induces platelet aggregation [42].

5.3. Dynamic Condition Experiment - MESH Experiment

The dynamic test was executed to prove the effect of an exogenous thrombus as adjuvant to platelet activation and thrombosis in presence of shear stresses. The obtained results for PAS assay have been shown in the previous chapter and they assess an overall activation for platelets at each time point and for all the tested velocities.

Tests have been performed at two different velocities for the pump (40rpm and 200rpm). The purpose of increasing the flow rate is to stimulate platelets with higher convective effects and to improve the contact between platelets and the thrombus.

For all the considered cases, two control samples have been taken into consideration. Control1 corresponds to the PAS measurement for stationary platelets inserted into the circuit and not interfacing any clot. It is useful to demonstrate that platelets contained in the GFP solution are not originally activated. Control2, instead, corresponds to the PAS value for platelets inserted into the circuit, flowing at the considered velocity set for the
pump (40rpm or 200rpm) and not interfacing any clot. The second control is instead necessary to verify the effect of the pump on the activation of platelets.

Figure 4.9., showing PAS values for platelets been in contact both with PRP clot and WB clot at 40rpm, confirms the phenomenon described for static test results. In fact, in the majority of cases, PAS data for tested samples differ significantly from both the control samples. This difference confirms the main hypothesis for a pump velocity of 40rpm, demonstrating that the thrombus has a procoagulant effect on platelets.

The pump is demonstrated not to activate platelets as the value of PAS for Control2 is not statistically different form the PAS value for Control1 at any time point.

Samples tested with WB thrombus load do not show significant differences from samples tested with PRP thrombus load. Therefore, the difference between the two type of thrombus described for the static test is not visible for the dynamic one. It is likely that platelets in the dynamic test, being able to flow in the loop, get easily in contact with both PRP and WB thrombus load, even if the latter is dry.

As seen for static results, platelet activation is observed in the first minute of the experiment even for dynamic tests. Curves represented in Figure 4.10., in fact, have a relevant slope at the beginning of the test, while afterwards activation rate is very slight, both for curves referred to sample tested with PRP and WB clots. Results can be read as a confirmation of the immediacy of the phenomenon: platelet activation occurs as platelets get in contact with the thrombus. In the first minute of the test the majority of cells touches the clot for the first time, resulting in a relevant activation rate, afterwards only platelets which have not interfaced the clot at the beginning are activated.

In order to test platelet activation with increased convective effects, the same experiment is executed at a higher velocity for the pump. Figure 4.11. and 4.12 show the obtained results.

PAS results are very similar to the ones obtained for test executed at 40rpm. In fact, the pump seems not to activate platelets as there is no difference between PAS values related to the two control samples at each time point. The activation induced by the presence of a red or a white thrombus load is confirmed. In fact, in the majority of cases, there is a statistically relevant difference between the tested samples and both the control samples.
This activation quickly increases at the beginning of the experiment when the majority of platelets gets in contact with the thrombus, whereas afterwards there is a slower increase of activation rate. As it was described for tests at 40rpm, even at 200rpm similar PAS values have been obtained for both samples tested with WB and PRP clots.

By comparing results obtained for samples tested at both the velocities, it can be said that the presence of a thrombus load, both red and white, interfacing the platelets implies an increase in platelet activation independently on the velocity at which they are flowing. Therefore, the higher velocity of the pump, introduced to improve the contact between thrombus and platelets, by increasing convective effects and by increasing the number of turns platelets realize in the same period, does not induce, as initially supposed, an increase in platelet activation. Therefore, this observation suggests that the introduction of convective effects at low velocity is sufficient to saturate the phenomenon and the increment in velocity and in convection does not induce further activation.

From all the obtained results, the effect of white and red thrombus loads on platelet activation is undeniable. Furthermore, it is very likely that platelet activation is immediate, platelets get activated as they get in contact with the load. So, the phenomenon occurs faster at the beginning of the experiment, when the majority of platelets interfaces the load for the first time and it occurs in a slower way afterwards as lower is the number of platelets that have not interfaced the clot. However, unfortunately, it is not possible to explain all the aspects of the phenomenon because of the high number of variables taking part in it and because of the complex, unpredictable and not always repeatable dynamic of the events happening in the circuit as the experiment begins.

Similar evaluations can be made about the comparison between samples tested with WB and PRP clots. The PAS values result to be similar in the two cases at each time point, but it is difficult to assert the exact dynamic of what happens to a dry and a damp clot being washed by a platelet solution at various velocities.

Both in static and dynamic experiments, platelet activation induced by the presence of a thrombus load has been demonstrated. However, platelets tested in static conditions have
shown higher PAS values with respect to the data obtained for the dynamic test. This is probably due to the different volumes of GFP solution (4ml for static test and about 10ml for dynamic test) which have been interfaced with the thrombus in the two cases. Therefore PAS results higher for the static test because the number of platelets interfacing the same amount of clot is lower than the one of platelets interfacing the clot in dynamic conditions.

As already observed, the activation phenomenon seems to occur, for both the test conditions, at the beginning of the experiments and this is visible from the plateau phase of the majority of the represented curves in the graphs. When the plateau phase is not really evident, it means that platelets have interfaced the thrombus load with different timing probably because of the experimental set-up.

5.4. Dynamic Condition Experiment with VAD

The dynamic test with VAD is executed to prove the effect of an exogenous thrombus as adjuvant to platelet activation and thrombosis with conditions as close as possible to the clinical situation. This test has to be considered as a pilot study which describes the phenomenon at a higher realistic level even though, because of differences in the circuit used, in the volumetric ratio of clot over GFP, in velocities and in the generated shear stresses, it is not quantitatively comparable with the static and the dynamic tests already discussed.

Results are shown in the previous chapter in Figure 4.13. and 4.14. and they confirm the overall hypothesis of increase in platelet activation due to the presence of a thrombus load interfacing platelets.

For all the considered cases, two control samples have been taken into consideration. Control1 corresponds to the PAS measurement for platelets flowing in the circuit. It is useful to verify the effect of the VAD on the activation of platelets. Control2, instead, corresponds to the PAS value for platelets flowing in the circuit and interfacing a clean mesh. This result is useful to verify the effects of the mesh on activation.

PAS values for Control1 are similar over time at the beginning of the test but they become higher at the end of it. It is possible to deduce that the LVAD HA5 has caused a slight increase in activation at the last time point of the experiment.
The statistical analysis stresses a difference between the two control samples at 30min and 60min since the beginning of the experiment. Therefore, the stainless steel mesh could be an adjuvant for platelet activation; this effect is not observed in the first part of the experiment but it is observed at the end of the test.

The mesh may cause further platelet activation for two main reasons: fluid dynamic effects due to the presence of the mesh or effects of the mesh material on platelets.

The presence of the mesh inserted in the circuit, in fact, may have caused a non-physiological flow with vortexes or areas of stasis, leading to local phenomena of increased shear stress. Although platelets are possibly exposed to this disturbed fluid dynamics for a time that is a small fraction of the total duration of the test, the entity of these effects may still be relevant and result in an increase of platelet activation.

The effects of the mesh on platelet activation could be caused even by the material itself. Actually a few studies, especially regarding the optimization of materials used for manufacturing stents, are found in literature. Their main aim is to improve the compatibility of stainless steel stents by coating them with layer of various origin. Furuzanmehr et al. [43], in 2013, demonstrated the improvement in biocompatibility by applying a coating of TiO$_2$ on the surface of the stent. Platelet activation was studied by valuating the expression of CD62P by flow cytometry. Coated surfaced of stents resulted to induce less activation for platelets. In 2015, Xiao et al. [44], modified the surface of stents in 316 stainless steel by grafting methoxy poly ethylene glycol. The surface-modified steel resulted to be more biocompatible and to induce less adhesion and activation for platelets.

Lucy di Silvio, in 2009, affirmed the possibility of ion release from bare stainless steel surfaces of devices. In her study the coating is considered positive, besides for improve the risk of high adhesion and activation, even to avoid this drawback [45].

Therefore, it is possible that the activation of platelets due to the presence of the mesh is caused by the interface between cells and stainless steel.

The present project has a few limits related to the lack in test aiming at clarify these effects. In fact, tests to observe the fluid dynamics of the flow and tests to assess the
contribute on activation of the mesh itself, in static conditions, could be useful to quantify and to better describe these phenomena.

Anyways, the statistical analysis confirms a significant difference between PAS values for PRP sample and PAS value executed for Control2 at 120min. Differences with a significance level not higher than 0.1 are observed between PAS values of these two samples at all the other time points. As p-values are really close to 0.05 and the variability of data is relevant, these differences are considered reliable, although they are not statistical. Therefore, it can be affirmed that the thrombus load do cause activation for platelets independently on the presence of the stainless steel mesh.

Probably because of the high number of platelets interfacing the clot, the phenomenon does not saturate over time. The volume of GFP solution is really high, compared to the volume of thrombus (about 90 ml of GFP solution vs. 120µl of thrombus load). Therefore, at the end of the experiments the number of platelets getting in contact with the clot is still relevant.

As the VAD loop has been inserted in the incubator at 37° for the entire duration of the experiment, the increase of temperature is just a replication of what happens to a VAD in vivo, with the difference that in vivo the device is surrounded by tissues, which retain heat, and not by air as in the incubator.

This variation of temperature explains the presence, reported in literature, of burned tissues in the proximity of the device can be easily explained. The increasing of temperature may affect, in some ways, platelets but it seems not to induce platelet activation. Maurer-Spurej and colleagues [46], in fact, demonstrated that platelets are not activated by high values of temperature but they become rather activated at low temperatures.

In any case, the possible effect of high temperature would be systematic for all the samples tested at a determined time point. Therefore, differences between samples collected at the same time are not affected by the variation of temperature.
Chapter 6

Conclusions and Future Directions

The present study aims at better understanding the effects of VAD-related thrombosis on the pro-thrombotic state in continuous flow ventricular assist devices. In particular, the impact of an exogenous thrombus load acting as an adjuvant to cause platelet activation and, therefore, VAD thrombosis has been examined.

As platelets are the main cellular factor which is involved in the coagulation process and in the formation of a thrombus load, these cells are the crux to understand this phenomenon and its consequences. In fact, activated platelets lead to further activation and aggregation of platelets with consequent further formation of thrombi.

This study has demonstrated that the presence of a clot interfacing platelets activates them and amplifies the activation phenomenon. Thus, VAD-related thrombosis induces activation of platelets leading to an amplification of the thrombotic phenomenon itself.

This sequence of events may be correlated with the fact that VAD-related thrombosis is usually an acute complication of the device, it occurs in a short time and it is unpredictable often leading to fatal consequences. In fact a thrombus load, even with a small size, may be a powerful platelet activator and, thus, amplify the phenomenon of thrombosis.

Platelet activation has been observed for all the tested conditions. Platelets interface the thrombus load and the activation phenomenon occurs quite immediately. The mechanism of activation is due to the contact between the two parts.

Thrombin is considered to be the main agonist for the activation of platelets. In the tests considered for this project, thrombin may still be expressed on the fibrin net of the thrombi or it may be generated by the TF which flows, in a small part, in blood. The presence of thrombin is independent on the cellular component forming the clot but it is rather related to the fibrous and protein-based component of the thrombus which is similar for both red and white clots. It may be for this reason that WB and PRP thrombi have the same influence on platelets activation.

Moreover, activation resulted to be proportional to the quantity of thrombus load which interfaces platelets, as it was evident in the static test.
The project has a few limitations.

In fact, although the effect of thrombosis on platelet activation has been clearly demonstrated, it is not possible to explain all the aspects of the phenomenon because of the high number of variables taking part in it and because of the complex, unpredictable and not distinguishable dynamic of the events that happen as the experiment begins. In fact, the thrombus load inserted in the set-up is washed by the platelet solution and it is partially removed from the substrate. The different consistence of the two types of clot increases the complexity of the phenomenon.

Further studies and analysis should be executed to improve the experimental set-up and to allow in-depth comparisons.

The obtained results for the dynamic tests have shown an overall activation both for platelets tested with WB and for platelets tested with PRP. The protocol used for the static experiments, which did not allow the proper movement of platelets in the tube inducing low platelet activation, was probably not optimal to achieve the supposed data.

The differences between the experimental conditions in which static and dynamic tests are performed and the ones related to the dynamic test in presence of a VAD, do not allow quantitative comparisons among all of them. This is considered another limitation of the present study that could be avoided by projecting a new adjusted set-up.

As the number of experiments for all the experiments is not very high, the repetition of the same tests would reduce the length of the error bars and allow to obtain more precise results.

From the data obtained for the dynamic test in presence of a VAD, the presence of the mesh interfacing platelets seemed to activate platelets. The possible reasons for this activation are related to local fluid dynamic effects in the nearness of the mesh or to the material of the mesh itself which may affect platelets.

To better understand the phenomenon and clarify its causes, a few types of experiment may be executed. For example, an experimental set-up for VAD experiments, that allows to monitor the fluid dynamics, the flow rate and to replicate the systematic impedances of the patient, may be projected. It would be useful to evaluate and simulate the fluid dynamics of the circuit. Moreover, the potential contact-related activation given by the mesh in static conditions could be useful to assess if the material of the mesh itself introduces a bias in the data. For the dynamic test in absence of VAD a further control,
which measures PAS for GFP flowing in the circuit interfacing a clean mesh, could give more information about the influence of the mesh on platelets.

In relation to the increment of the temperature registered for the dynamic experiment with VAD, in case the influence on platelets of changes in temperature is confirmed, experiments which test platelets in the same situation but at constant temperature may be useful to avoid the effect of increasing temperature on platelet activation.

In case of necessity, other methods, such as flow cytometry and the evaluation of concentration of platelet microparticles, the examination of platelet aggregate or the evaluation of P-selectin levels, may provide further confirmations of platelet activation.

Thrombosis related to VAD implantation is a very complex pathology which still needs to be fully understood and clarifies. With the final aim of device improvement and life-saving innovation, the present project focused on the relevant role of platelets in inducing thrombosis, once they are stimulated by a chemical agonist. The demonstrated activation of cells due to their contact with a thrombus load is important to understand the dynamic of the thrombotic phenomenon and to develop new strategies to deal with this situation.

The final purpose of the entire research group is to contribute to increment the knowledge of these devices in order to develop optimized VADs with less thrombotic effects on blood and thus improving the survival and the life quality of patients.
References

1. www.nhlbi.nih.gov
3. www.heartstroke.com
4. www.medicinenet.com
5. www.heart.org
6. www.ccmdweb.org
7. www.emsworld.com
12. www.ucsfhealth.org
14. www.texasheart.org
15. www.vadsupplies.com
17. www.thebiochemistrypage.org
18. www.levenmethemofilie.be
19. www.platelets.se
31. Chiu W., Thrombus formation pattern in the HeartMate II VAD-Clinical observation can be predicted by numerical simulations, ASAIO Journal, 2014, 60(2):237-240.


