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Experimental analysis of hemocompatibility-related adverse events in the setting of LVAD support: the competing role of von Willebrand factor disease, prothrombotic platelet activation and antithrombotic therapy

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List of Acronyms

Ac-FII	Acetylated-FII
ACD-A	Anticoagulant Citrate Dextrose Solution A
aHF	Advance Heart Failure
ASA	Acetylsalicylic Acid
AT	Antithrombotic
AT-III	Antithrombin III
avWS	Acquired von Willebrand Syndrome
BTT	Bridge to Transplant
CB	Collagen Binding
CF-LVAD	Continuous Flow-Left Ventricular Assist Device
CH-TH	Chromozin-TH
COX	Cyclooxygenase
DD	D-Dimer
DT	Destination Therapy
ECMO	Extra-Corporeal Membrane Oxygenator
ELISA	Enzyme-linked Immunosorbent Assay
FC	Flow Cytometry
FDA	Food and Drug Administration
FG	Fibrinogen
GFP	Gel-Filtrated Platelet
HB	Hemoglobin
HF	Heart Failure
HT	Hematocrit
HVAD	HeartWare Ventricular Assist Device
HM	HeartMate
IABP	Intra-Aortic Balloon Pump
INR	International Normalized Ratio
INTERMACS	Interagency Registry of Mechanically Assisted Circulatory Support
LDH	Lactate Dehydrogenase

LTA	Light Transmission Aggregometry
LVAD	Left Ventricular Assist Device
MCS	Mechanical Circulatory Support
PA	Platelet Activation
PAS	Platelet Activity State
PB	Platelet Buffer
PFA	Platelet Function Analysis
PFP	Platelet Free Plasma
PG	Prostaglandin
PI	Pulsatility Index
PLT	Platelet
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
ROTEM	Rotational Thromboelastometry
TF	Tissue Factor
TEG	Thromboelastography
TGT	Thrombin Generation Test
TX	Thromboxane
VAD	Ventricular Assist Device
vWf	von Willebrand Factor

Abstract

Background: Advanced Heart Failure (aHF) represents the advanced phase of multiple pathological conditions of the heart and coronary arteries. This condition leads to a compromised heart function and, consequently, to an inadequate cardiac output. To date, preventive measures and medical therapies are not able to contrast and/or reduce aHF morbidity and mortality. The only resolutive therapy is heart transplant but the demand for the organ has exceeded the availability of donor heart and there are rigid selection criteria for transplant candidates[2].

Thanks to bioengineering's developments over the last 50 years, mechanical circulatory support (MCS) devices are today an effective solution for the treatment of patients affected by aHF. Among these, the most suitable devices for long-term treatment are ventricular support devices (VAD), in particular continuous flow VAD for the left ventricle (CF-LVAD). LVADs are electrical miniaturized mechanical pumps with an internal rotating impeller (axial or centrifugal) that provides the propulsive energy needed to pump a continuous flow of blood from the apex of the left ventricle to the aorta. Accordingly, the device mechanically supports the pumping function of the left ventricle and, in the most severe pathologies, completely replaces it[3,4].

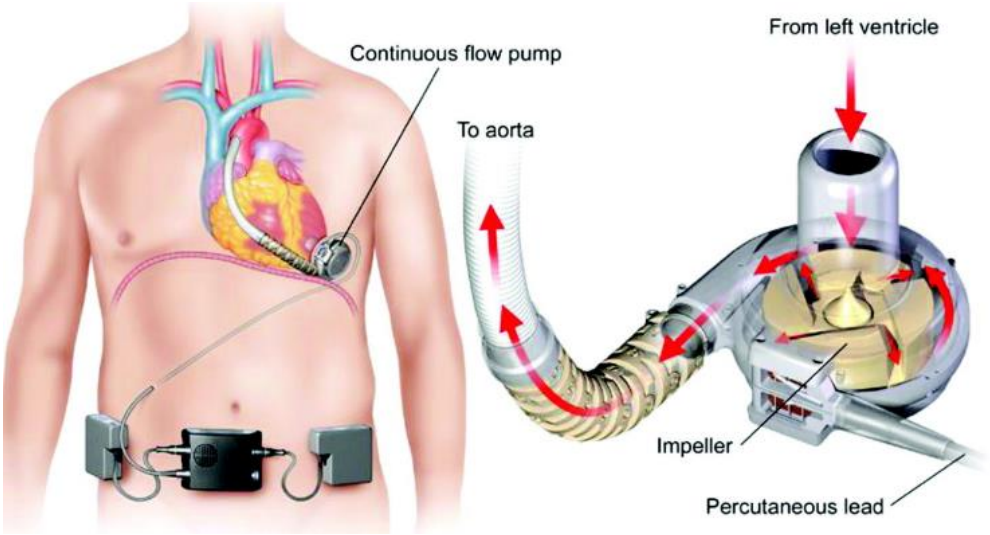


Figure I – Functional components of CF-LVAD.

Despite latest generation CF-LVADs demonstrated significant technological progress, which assured a progressive increase in patient survival and overall outcome improvement, LVAD therapy remains plagued by post-implant complications due to the non-physiological pump-patient interaction. In particular hemocompatibility-related adverse events (HAREs, namely thrombosis and bleeding) severely affect long-term outcomes[5,6].

Thrombotic complications (pump thrombosis or ischemic stroke), besides the interaction between blood components and device surface, can be principally attributed to the non-physiologic blood flow patterns characterizing the device. Indeed, LVADs operate at very high rotational speeds to achieve physiological cardiac outputs, which generate high turbulent and non-physiological shear-stress levels (100 – 500 Pa). As a result, platelets are exposed to elevated mechanical loading, which acts as agonist for platelet activation (PA), leading to thrombus formation[7,8,9]. In the same way, bleeding complications are determined by the enzymatic degradation of von Willebrand factor (vWf), translating in the loss of its high-molecular weight (HMW) multimers, caused by the supraphysiological shear stress imposed by the pump. The qualitative and quantitative deficiency of these multimers reflects a deficit in factor's functional activity[10,11,12].

In order to limit the prothrombotic platelet activity and prevent thrombosis, LVAD patients are routinely administered with a combination of antiplatelets and antithrombotic (AT) drugs (usually Aspirin (ASA) and Warfarin, respectively). Nonetheless, AT therapy do not totally eliminate the risk of thrombosis as a consequence of the so-called drug "resistance", which includes a portion of patients who are insensitive to drug loading. Moreover, the lack of a patient-specific AT regimen could lead to under-coagulation, enhancing pump thrombosis, or to over-coagulation, which induces, in turn, bleeding events[13,14,15].

To date, few studies focused on the fragile balance between the factors responsible for hemorrhagic and thrombotic events.

The present study is part of a research project born from the collaboration between the Biomechanics group of the Department of Electronics, Information and Bioengineering (DEIB) of Politecnico di Milano and Vita Salute San Raffaele University (UniSR) of Milan, and aims at experimentally analyzing the competing role of i) von Willebrand factor disease, ii) platelet activation, and iii) antithrombotic therapy to the occurrence of HRAEs in LVAD patients.

Materials and Methods: Experimental activities have been performed into the Haemostasis and Thrombosis Research Laboratory of San Raffaele Hospital (HSR). The Platelet Activity State (PAS) assay was used to evaluate the platelet prothrombotic activity whereas an Enzyme-linked Immunosorbent Assay (ELISA) evaluated the degradation profile of vWf. Moreover, standard coagulation parameters (hematocrit, hemoglobin, platelet count, INR, aPTT, fibrinogen and D-Dimer) and occurrence of HRAEs were recorded, and clinical outcomes correlated to the antithrombotic regimen. Patients were classified in two groups: patients who did not develop a bleeding event (control group) and patients who did (study group). Data were compared at three different time points: T0 (pre-implant), T1 (early-term follow up) and T2 (long-term follow-up); in addition, consistent with the change in antithrombotic therapy (ASA discontinuation) that followed a bleeding event, data from patients of the study group were compared pre- and post- ASA discontinuation.

The experimental protocol for the PAS assay requires the collection of platelet rich plasma (PRP) from a whole blood sample taken from a cf-LVAD patient. The PRP is filtered through a gel-filtration column to obtain a suspension of platelets (Gel-Filtered Platelets, GFP) free of plasma components, which is then diluted to a constant concentration of 20.000 plt/μL. The platelets are then exposed to Ac-FII for 10 minutes at 37 °C – in addition to FXa and Ca2+ needed to rebuild the prothrombinase complex – to quantify the thrombin production rate as a marker of PA. The amount of thrombin generated from the GFP sample is quantified by spectrophotometric analysis, where chromozyn-TH is the thrombin-specific chromogenic peptide substrate. The spectrophotometer detects the dynamics of absorbance values variation over a predetermined time interval: the slope of the line that interpolates the absorbance-time data points is an index of the thrombin quantity produced by the platelet sample. The analysis is performed both on a non-stimulated GFP sample and on a sonicated GFP sample (for 10 seconds, 20 W). The PA level of the subject (PAS value) is expressed as a percentage of its maximum potential of activation – i.e. PAS value obtained from the sonicated sample. For each patient, the PAS assay has been performed in duplicate to increase data consistency.

ELISA experimental protocol consists in a series of steps to measure the ability of von Willebrand factor to bind collagen (vWf:CB). This ability is strictly related to the presence pf vWf HMW multimers. A reduced rate of vWf collagen binding activity is an indicator of the loss of these multimers and, consequently, to a degraded profile of the factor. The test

principle regards the measurement of vWf, which is captured through HMW multimers by human collagen coated on the internal walls of a plastic microplate well. Then, rabbit anti-vWf antibodies coupled with peroxidase bind to the remaining free antigens of the bound vWf. The bound enzyme peroxidase is revealed by the addition of tetramethylbenzidine (TMB) substrate. The intensity of the color read by a spectrophotometer is directly proportional to the initial concentration of vWf in the plasma sample.

Results: The study population comprises 45 patients implanted with a CF-LVAD with the indication for destination therapy. Of these, 17 (38%) are in the control group and 28 (62%) in the study group. In the control group, 2 (12%) patients suffered a thrombotic event. In the study group, bleeding events were followed by ASA withdrawal for 26 (93%) out of 28 patients; before ASA suspension, 1 (4%) patient suffered a thrombotic event whereas after ASA suspension 5 (19%) patients developed a bleeding event and 1 (4%) a thrombotic event. Considering the two tested groups (control vs. study), analyzes were carried to compare both vWf collagen binding activity and PAS values. Regarding the first factor, the overall population is subjected to a statistically significant reduced vWf activity over time of LVAD support. However, no differences were detected between the two groups. Similarly, analysis of vWf:CB pre- vs. post- ASA discontinuation did not reveal a significant difference.
Regarding PAS values, no significant changes were registered in the overall population. Similarly, no statistically significant changes were noted between the two groups. On the other hand, ASA suspension induced a significant - but low-magnitude - increase in PAS values.
In addition, the sub-analysis related to the different pump models implanted (HM3 vs. HVAD) showed no statistically variations in both vWf:CB and PAS values.

Conclusions: Experimental analyzes of the factors responsible for HRAEs in LVAD patients suggest that patients who suffer from thrombotic and bleeding complications are characterized by comparable profile of PA (consistent with no significant differences in PAS values) and degradation of vWf HMW multimers. Interestingly, a variation in the antithrombotic pharmacological therapy may effectively contribute to reduce the incidence of hemorrhagic events and, in parallel, not significantly increase the thrombotic risk. Moreover, the results of this study highlight the need of tailoring the antithrombotic strategy

to patient-specific hemostatic profile; in this perspective, combining constant monitoring of PA and patients clinical status, emerge as a valuable strategy to preserve the delicate balance within the two arms of coagulation complications (hemorrhagic and thromboembolic).

Keywords: LVAD, hemocompatiblity, von Willebrand factor, platelet activation, antithrombotic therapy, bleeding, thrombosis.

Sommario

Introduzione: L'insufficienza cardiaca avanzata (advanced Heart Failure, aHF) rappresenta la fase inoltrata di molteplici condizioni patologiche del muscolo cardiaco e delle arterie coronarie. Questa condizione conduce ad uno stato alterato delle funzionalità del cuore e, di conseguenza, ad una inadeguata portata cardiaca. Ad oggi, le misure preventive e le terapie mediche non sono in grado di contrastare e/o ridurre la morbosità e la mortalità associate all'insufficienza cardiaca avanzata. L'unica terapia risolutiva è il trapianto cardiaco, tuttavia la richiesta supera di gran lunga la disponibilità di donatori e i candidati al trapianto sono soggetti a rigidi criteri di selezione[2].

Grazie alle innovazioni degli ultimi 50 anni nel campo dell'ingegneria biomedica, i dispositivi meccanici di supporto circolatorio (Mechanical Circulatory Support devices, MCS devices) rappresentano oggi una valida soluzione per il trattamento di pazienti affetti da aHF. Tra questi, i dispositivi che si adattano maggiormente al trattamento a lungo termine sono i dispositivi di assistenza ventricolare (Ventricular Assist Devices, VAD), in particolare i dispositivi VAD a flusso continuo per il supporto funzionale del ventricolo sinistro (Continous Flow-Left Ventricular Assist Devices, CF-LVADs). Allo stato dell'arte, questi dispositivi sono pompe miniaturizzate impiantabili, alimentate elettricamente, all'interno dei quali un impeller rotante (assiale o centrifugo) fornisce l'energia propulsiva necessaria a direzionare il flusso continuo di sangue dall'apice del ventricolo sinistro all'aorta. In questo modo, il dispositivo fornisce un supporto meccanico all'azione di pompa del ventricolo sinistro e, nei casi di patologie più severe, la sostituisce completamente[3,4].

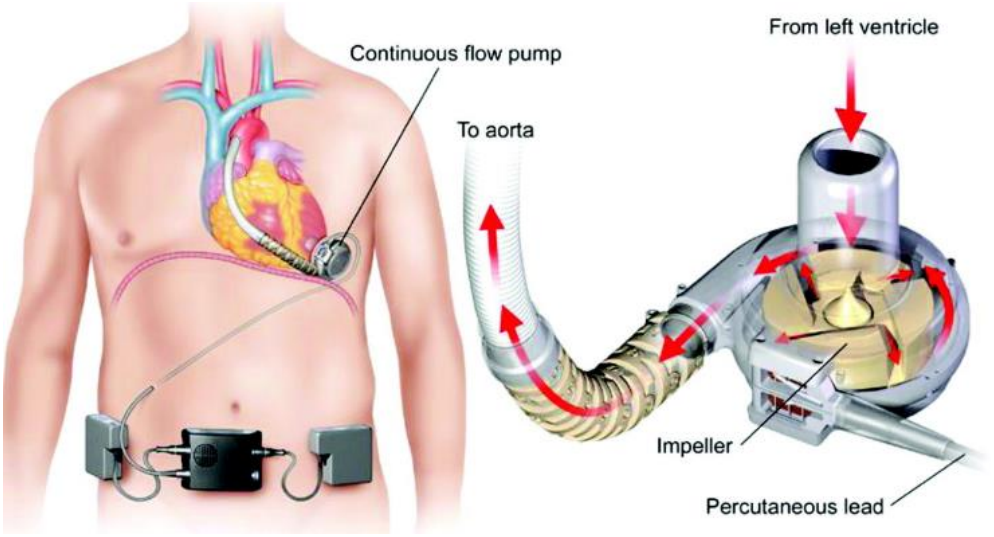


Figure I – Componenti funzionali di CF-LVAD.

Nonostante gli LVAD a flusso continuo di ultima generazione abbiano dimostrato un grande progresso tecnologico, il quale ha permesso un considerevole aumento della prospettiva e della qualità di vita dei pazienti, questo tipo di supporto presenta severe complicanze post-impianto dovute all'interazione non fisiologica tra pompa e flusso sanguigno[5,6]. Le complicanze trombotiche (trombosi della pompa o ictus ischemico), oltre all'interazione tra le componenti ematiche ed il dispositivo, sono principalmente attribuite al flusso emodinamico non fisiologico imposto dalla pompa. Infatti, CF-LVAD opera ad elevate velocità di rotazione dell'impeller della pompa, necessarie a garantire portate sanguigne fisiologiche, che generano elevati e non fisiologici sforzi di taglio (100-500 Pa). Questi ultimi agiscono come agonisti dell'attivazione piastrinica, componente fondamentale nella generazione del trombo[7,8,9]. Allo stesso modo, le complicanze emorragiche dipendono dagli elevati sforzi di taglio che determinano una progressiva degradazione enzimatica del fattore di von Willebrand (von Willebrand factor, vWf) che si traduce nella perdita di multimeri ad alto peso molecolare (high-molecular weight multimers, HMWMs); la carenza di questi multimeri comporta una riduzione dell'attività funzionale del fattore[10,11,12].

Per limitare l'attività protrombotica delle piastrine e prevenire l'insorgenza di trombi, ai pazienti portatori di LVAD viene normalmente somministrata una terapia combinata di farmaci antipiastrinici ed antitrombotici (in genere Warfarin ed Aspirina (ASA), rispettivamente). Tuttavia, la terapia non elimina completamente il rischio trombotico, specialmente nel caso si sviluppasse una resistenza al farmaco, condizione riguardante gruppi di pazienti in cui l'efficacia del farmaco risulta notevolmente ridotta o inesistente. In

aggiunta, l'assenza di regimi antitrombotici paziente-specifici possono comportare situazioni di ipocoagulazione, con elevato rischio di trombosi della pompa, o di ipercoagulazione che, al contrario, può indurre emorragie[13,14,15].

Finora, la letteratura presenta pochi studi riguardanti la relazione che intercorre tra i fattori responsabili del rischio trombotico ed emorragico e la terapia antitrombotica somministrata. Il presente lavoro di tesi si inserisce in un progetto di ricerca nato dalla collaborazione tra il Gruppo di Biomeccanica del Dipartimento di Elettronica, Informazione e Bioingegneria (DEIB) del Politecnico di Milano e l'Università Vita-Salute San Raffaele (UniSR) di Milano. L'obiettivo dello studio riguarda l'analisi dell'azione competitiva tra la sindrome del fattore di von Willebrand acquisita, l'attivazione piastrinica e la terapia antitrombotica nell'occorrenza di eventi avversi (trombotici ed emorragici).

Materiali e Metodi: Le attività sperimentali sono state svolte presso il Laboratorio di Ricerca di Emostasi e Trombosi presso l'Ospedale San Raffaele (OSR). Sono stati utilizzati due saggi chimici per valutare, rispettivamente, l'attività protrombotica delle piastrine e il profilo di degradazione del vWf: Platelet Activity State (PAS) assay e Enzyme-linked Immunosorbent Assay (ELISA). Nello studio sono stati coinvolti pazienti portatori di CF-LVAD e sono stati suddivisi in due gruppi: pazienti che non hanno avuto un evento di sanguinamento (gruppo di controllo) e pazienti che ne hanno sofferto (gruppo di studio). I dati sono stati confrontati a tre differenti istanti temporali: T0 (pre-impianto), T1 (breve periodo post-impianto) e T2 (lungo periodo post-impianto); inoltre, i pazienti del gruppo di studio che hanno sospeso la terapia antitrombotica, a seguito dell'evento di sanguinamento, sono stati valutati anche nei periodi pre- e post-sospensione del farmaco (ASA). I risultati sono stati poi confrontati con i parametri clinici ed il profilo coagulativo di ciascun paziente, misurati durante la campagna sperimentale, così da avere un ulteriore confronto con lo stato coagulativo dei pazienti portatori di un CF-LVAD. Infine, per ciascun gruppo, sono stati registrati gli eventi avversi di natura trombotica ed emorragica.

Il protocollo sperimentale per il PAS assay prevede l'estrazione del plasma ricco di piastrine (PRP), in seguito a centrifugazione, da un campione di sangue intero prelevato da un paziente portatore di cf-LVAD. Il PRP viene filtrato mediante una colonna per filtrazione su gel per ottenere una sospensione di piastrine priva delle componenti plasmatiche (Gel-Filtered Platelets, GFP), che viene successivamente diluita fino a raggiungere una

concentrazione costante di 20.000 plt/μL. Le piastrine vengono quindi esposte al Ac-FII per 10 minuti a 37°C – in aggiunta di FXa e di Ca^{2+} necessari per ricostruire il complesso protrombinasi – al fine di quantificare la produzione di trombina, marcatore universalmente riconosciuto della PA. La quantità di trombina generata dal campione viene quantificata da un'analisi spettrofotometrica, grazie all'aggiunta della cromazina-TH come substrato peptidico cromogenico specifico per la trombina. Lo spettrofotometro rileva la dinamica di variazione del valore di assorbanza del campione durante un intervallo di tempo prestabilito (7 minuti): la pendenza della retta interpolante i punti assorbanza-tempo è indice della quantità di trombina prodotta. L'analisi viene eseguita sia su un campione di GFP non stimolato (basale) sia su un sonicato (per 10 secondi a 20 W). Il livello di PA del soggetto in esame (valore di PAS) viene espresso in termini percentuali rispetto al suo massimo potenziale di attivazione, ottenuto dal campione sonicato.

Il protocollo sperimentale per l'ELISA consiste in una serie di passaggi per misurare la capacità del vWf di legarsi al collagene (vWf collagen binding, vWf:CB). Questa funzione è strettamente connessa alla presenza di HMWM; pertanto, una ridotta attività del fattore verso il collagene rappresenta un indicatore della mancanza di questi multimeri e, conseguentemente, della sua degradazione. Il principio del test si basa sulla misurazione della concentrazione di vWf nel plasma testato, il quale si lega, mediante i suoi HMWM, al collagene umano adsorbito sul fondo dei pozzetti della piastra utilizzata. Successivamente, vengono incubati anticorpi specifici per il vWf, i quali sono coniugati ad un enzima (perossidasi) che consente la conversione del substrato da incolore a colorato. Questa conversione avviene mediante la reazione chimica tra l'enzima perossidasi e l'aggiunta di tetrametilbenzidina (TMB). L'intensità del colore sviluppato, letta mediante uno spettrofotometro, è direttamente proporzionale alla concentrazione iniziale di vWf nel campione di plasma testato.

Risultati: La popolazione di studio è composta da 45 pazienti impiantati con indicazione al trattamento DT. Di questi, 17 (38%) appartengono al gruppo di controllo e 28 (62%) al gruppo di studio. Nel gruppo di controllo, 2 (12%) pazienti hanno sviluppato un evento trombotico. Nel gruppo di studio, a seguito degli eventi di sanguinamento, 26 (93%) pazienti su 28 hanno sospeso il farmaco ASA; prima della sospensione, 1 (4%) paziente ha sofferto di un evento trombotico mentre dopo la sospensione 5 (19%) pazienti sono andati incontro

ad un evento di sanguinamento e 1 (4%) paziente ad uno trombotico. Considerando i due gruppi testati (controllo vs. studio), le analisi sono state condotte con l'intento di confrontare l'attività del vWf nel legarsi al collagene e i valori di PAS. Nel caso del vWf, la popolazione nel complesso ha mostrato una riduzione significativa dell'attività del fattore nel tempo. Al contrario, sia il confronto tra gruppi sia il confronto tra pre- e post-sospensione di ASA non hanno mostrato variazioni significative dei valori di vWf:CB. Osservando invece i valori di PAS, nessuna variazione statisticamente significativa nel tempo è stata registrata sia considerando la popolazione nel complesso sia effettuando un confronto tra i due gruppi. Al contrario, le due condizioni pre- e post-sospensione ASA hanno mostrato differenze significative in termini di PAS.
In aggiunta, è stata condotta una sub-analisi relativa ai diversi modelli di pompa impiantati nei pazienti testati (HM3 vs. HVAD); i risultati hanno mostrato che non vi è nessuna differenza statistica osservabile tra le due pompe sia per i valori di vWf:CB sia per quelli di PAS.

Conclusioni: Le analisi sperimentali condotte, considerando sia le cause emorragiche sia quelle trombotiche nei pazienti testati, suggeriscono che una variazione nella terapia farmacologica antitrombotica potrebbe contribuire alla riduzione dell'incidenza di eventi emorragici nel contesto di LVAD e, allo stesso tempo, non determinerebbe un aumento significativo del rischio trombotico. Si sottolinea inoltre la necessità di strategie farmacologiche paziente-specifiche che, abbinate ad un monitoraggio costante dell'attivazione piastrinica e dello stato clinico del paziente, contribuirebbero a preservare il delicato equilibrio esistente tra i due rami delle complicanze coagulative (emorragiche e tromboemboliche).

Parole chiave: LVAD, fattore di von Willebrand, attivazione piastrinica, terapia antitrombotica, sanguinamento, trombosi.

Introduction

The current work is part of a research project born from the collaboration between the Biomechanics group of the Department of Electronics, Information and Bioengineering (DEIB) of Politecnico di Milano and Vita Salute San Raffaele University (UniSR) of Milan. Among the clinical activities program of San Raffaele Hospital (HSR), UniSR promotes a study designed to analyze the phenomena associated to platelet function in patients with Left Ventricular Assist Device (LVAD) to treat advanced heart failure.

Experimental activities were conducted at the Platelet Activity State Laboratory (PAS Lab) of HSR in Milan.

Aim of the study is the analysis of the relationship between Von Willebrand factor (vWf) disease, prothrombotic platelet activation (PA), and antithrombotic therapy in LVAD patients to investigate their incidence in hemocompatibility-related adverse events (HRAEs) namely hemorrhagic and thromboembolic complications.

Two experimental protocols, which include a chemical assay, are utilized:

i. Platelet Activity State (PAS) assay. It quantifies thrombin production by platelets and correlates it with their level of activation, providing a pro-thrombotic profile of platelets
ii. Enzyme-linked immunosorbent assay (ELISA) for vWf collagen binding activity (vWf:CBA). It quantitatively determines the capacity of Von Willebrand factor to bind collagen, which is related to its functional activity

Patients plasmas needed for the tests were obtained from whole blood samples drawn from LVAD patients.

Data were compared between the two different groups of patients, divided according to the occurrence of a bleeding event:

i. Patients who did not develop a bleeding event (non-bleeding group)
ii. Patients who developed a bleeding event (bleeding group)

For each patient, vWf:CBA and PAS values were determined and evaluated at the following time points:

- Pre-implant (T_0), to define the patient-specific basal values

- Short-term follow up (T_1), related to a period < 3 months after the implant to monitor patient's response to device implantation and pharmacological therapy
- Long-term follow up (T_2), related to a period > 6 months after the implant to monitor patient's conditions
- In correspondence of a post-implant bleeding complication which determined a changing in pharmacological therapy, characterized by ASA suspension:
    - Short-term NO_ASA (T_1*), regarding a period < 3 months after the suspension of the drug
    - Long-term NO_ASA (T_2*), regarding a period > 6 months after the suspension of the drug

The thesis is structured in five chapters:

1. The first chapter describes the clinical and research fields characterizing the study. In detail, description of advanced heart failure and its consequent therapeutic treatments, focusing on LVAD technology, are presented. Then, continuous flow LVAD generation as HeartMate 3 and HeartWare HVAD are described, which are the devices currently implanted at HSR. Furthermore, principal post-implant complications are characterized, focusing on hemorrhagic and thrombotic events and, consequently, on the main factors contributing to their occurrence: platelets, Von Willebrand factor, and pharmacological therapy. In conclusion, platelets and vWf biological activity and functional alteration, following LVAD implantation, are described as well as the action of pharmacological therapy in the organism.
2. The second chapter describes the principal parameters currently utilized in clinics to evaluate coagulative and pro-thrombotic state of LVAD patients and to monitor the efficacy and the effects of pharmacological therapy. Then, laboratory tests commonly used in research, including PAS assay and ELISA vWf:CBA, are presented.
3. The third chapter concerns the description of patients involved in the study, the experimental protocols for PAS assay and ELISA vWf:CBA, and the statistical analysis applied.
4. The fourth chapter reports the results obtained from PAS assay and ELISA vWf:CBA in the two populations; then, clinical data are compared with results and the occurrence of hemorrhagic and thrombotic risks is evaluated.

5. The fifth chapter discusses the conclusions of the present study. Moreover, study limitations and future implications are presented.

Chapter 1

Heart failure, Mechanical Circulatory Support and Ventricular Assist Devices

Heart failure (HF) is a pervasive condition with high morbidity and mortality with an estimated prevalence of >37.7 million individuals globally[2]. The American College of Cardiology/American Health Association (ACC/AHA) have classified HF in four stages related to the state of the disease (Table 1.1)[16], defining it as "a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood". The earlier stages (A and B) of the disease are characterized by mild symptoms but patients are at high risk of suffering from refractory or symptomatic disease. As the course of the pathology progresses at stage C, ventricular activity is maintained by compensatory mechanisms – neurohumoral and cytokine systems, adrenergic stimulation – that become less effective over time until the cardiac function deteriorates and patients have marked symptoms at rest (stage D).

Stage	Description
A – high risk of developing HF	Hypertension, diabetes mellitus, family history of cardiomyopathy, CAD
B – asymptomatic HF	Previous MI, LV dysfunction, valvular heart
C – symptomatic HF	Structural heart disease, dyspnoea and fatigue, impaired exercise tolerance
D – refractory end-stage HF	Marked symptoms at rest despite maximal medical therapy

Table 1.1 – ACC/AHA classification of Chronic Heart Failure[16]. Abbreviations: CAD, coronary artery disease; MI, myocardial infarction; LV, left ventricle.

Heart transplantation remains the "gold-standard", as definitive treatment, for patients with refractory end-stage heart failure. However, the demand for the organ has exceeded the availability of donor hearts. For this reason, the need for mechanical circulatory support

(MCS) has become crucial[3]. MCS includes a wide variety of devices that can assure shot- or long-term support with the aim of providing coronary, cerebral, and systemic perfusion with effective left ventricular decompression.

Short-term MCS is used for acute HF to rest the ventricle and to allow the recovery of its function. Numerous studies have shown in fact that myocardium is able to repair itself during a period of unloading and, after that, patients experience an improvement in the quality of life[16]. Examples of these devices are (Image 1.1):

- Intra-Aortic Balloon Pump (IABP), used frequently in high risk cardiac surgery – typically for acute coronary ischemia related to critical coronary lesions. Balloon inflation during diastole improves coronary, cerebral, renal, and mesenteric blood flow. Balloon deflation during systole reduces left ventricular (LV) afterload, left ventricular end-diastolic pressure, right ventricular afterload, and reduction in myocardial oxygen demand. The overall effect results in an improvement in LV contractility and stroke volume[17].
- Extracorporeal Membrane Oxygenation (ECMO), it provides full hemodynamic support and can completely substitute the function of the failing heart during a cardiogenic shock. Through a venous cannula placed in central veins, the system draws out venous blood from the patient, removes carbon dioxide and replaces lung function through the oxygenation of blood, then pumps back the blood into the arterial system using a centrifugal pump via a short arterial cannula placed in an artery[18].
- Percutaneous Ventricular Assist Devices (pVAD) like IMPELLA®, axial flow miniaturized pump positioned on a catheter to pump blood from the left ventricle, across the aortic valve, into the aortic root. It contains a single cannula located in the aortic valve that performs both the inflow and outflow functions. As results, it reduces LV load, end-diastolic pressure, oxygen consumption, and myocardial work[19].

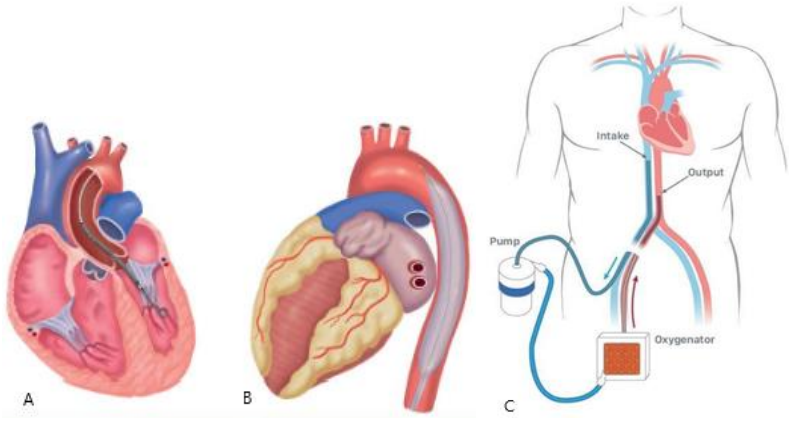


Image 1.1 – Schematic drawings showing mechanical circulatory support devices: (**A**) Impella, (**B**) IABP[20], (**C**) ECMO[21].

In chronic acute HF, long-term MSC is preferred to have a prolongate restoration of myocardial function. Durable MCS can be classified as implantable Ventricular Assist Devices (VAD) or Total Artificial Heart (TAH)[3].

The general goals of device therapy were defined by the Centers for Medicare and Medicaid Services (CMS) in 1984 and they have been classified into five categories as shown in Table 1.2. Patients can shift from one category to another depending on their conditions and on how their body responds to the administered therapy. In general, MCS device therapy is reserved for patients at stage D of HF with reduced ejection fraction[3].

Goal of therapy	Definition
Bridge to Transplant (BTT)	Transplant-eligible patients who otherwise would not survive before a donor heart becomes available
Destination Therapy (DT)	Patients who require lifelong MCS as an alternative to heart transplantation
Bridge to Candidancy (BTC)	Patients who are not currently transplant-eligible but who do not have absolute contraindications and who may be reconsidered for transplant after a period of temporary circulatory support
Bridge to Recovery (BTR)	Patients who require a temporary period of MCS for cardiac recovery from acute insults such as cardiogenic shock
Bridge to Decision (BTD)	Patients who are unassigned at device implantation

Table 1.2 – Goals of therapy in long-term MCS therapy candidates[3].

Several scoring systems have been designed for prognostication in HF patients to select objectively the MSC device therapy. The most useful empiric data derives from INTERMACS which contains over a decade of data collected from 15,000 patients and 158 hospitals in North America. It has been established in 2006 as a collaboration between National Heart, Lung, and Blood Institute (NHLBI), hospitals, and MCS industry and it is a classification system composed by seven levels (Table 1.3) to describe the preoperative condition of end-stage HF patients[3].

INTERMACS 1	Critical cardiogenic shock "crash and burn"
INTERMACS 2	Progressive decline "sliding on inotropes"
INTERMACS 3	Stable but inotrope dependent "dependent stability"
INTERMACS 4	Resting symptoms
INTERMACS 5	Exertion intolerant
INTERMACS 6	Exertion limited
INTERMACS 7	Advanced NYHA III, living comfortably limited to mild exertion

Table 1.3 – INTERMACS profile description[4]. NYHA, New York Heart Association.

For patients with advanced HF, implantation of a durable VAD has emerged as a bridge to transplant or as destination therapy for those who are ineligible for transplant. Moreover, ventricular assist devices can be a bridge to decision for patients who are ineligible for transplant at the time of implantation but may become eligible after the procedure and may also be utilized to promote myocardial recovery in a bridge to recovery strategy. Clinical trials have revealed the ability of the VAD to provide adequate support in both the BTT and DT setting[22].

This study focuses the attention on implantable VADs, especially on Left Ventricular Assist Devices (LVADs), which support the functionality of the left ventricle. They are electrical miniaturized mechanical pumps with an internal rotating impeller (axial or centrifugal) which provides the propulsive energy needed to pump a continuous flow of blood from the apex of the left ventricle to the aorta.

The first successful use of clinical LVAD was in 1966 when Dr. Debakey performed a double valve replacement in a 37-year-old female, who then developed post cardiotomy cardiogenic shock. He successfully implanted a pneumatically powered, paracorporeal

LVAD from the left atrium to the right subclavian artery. After 10 days of support, the patient recovered and the LVAD was removed. Then Dr. Norman implanted the first LVAD as a bridge to transplantation in 1978.

Since then, different randomized studies were conducted until the Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) trial of 2001 played a pivotal role in the approval of the LVAD as destination therapy in the US in November 2002[23].

There are five main components of an LVAD (Figure 1.2): i) an inflow cannula, ii) a pump, iii) an outflow cannula, iv) a percutaneous driveline, and v) an external controller. The inflow cannula is inserted into the apex of the left ventricle, and the outflow cannula is usually anastomosed to the ascending aorta. Blood returns from the lungs to the left side of the heart and exits through the left ventricular apex and across an inflow cannula into the prosthetic pumping chamber. Blood is then actively pumped through an outflow cannula into the ascending aorta. The pumping chamber is placed within the abdominal wall or peritoneal cavity. A percutaneous driveline carries the electrical cable and air vent to the battery packs and electronic controls that are worn on a shoulder holster and belt, respectively[24].

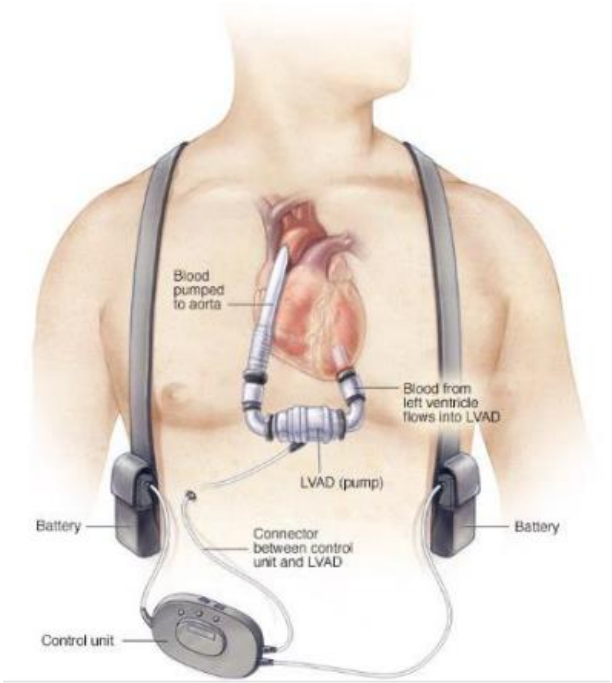


Figure 1.2 – Basic left ventricular assist device components[24].

LVADs can be classified based on their mechanical function: pulsatile flow (PF) or continuous flow (CF).

The first-generation LVADs (Figure 1.3A) rely on PF technology to simulate native cardiopulmonary circulation with volume-displacement chambers that would fill, then empty, either by pneumatic or electrical pump. These pumps have multiple moving parts, one-way valves, and a flexible pumping chamber, resulting in a complex system to be prone to breakdown and fail[23].

The second-generation (Figure 1.3B) relies on axial CF technology. The peculiar mechanical changes include the elimination of valves and chambers and the introduction of an internal rotor, which is suspended by contact bearings. However, the direct contact between blood and bearings promotes thrombosis[23].

The third-generation LVADs (Figure 1.3C) consist of centrifugal CF pumps with a technological advancement consisting in noncontact bearings using magnetic levitation. The centrifugal CF pump is designed to allow centrifugal flow of blood as opposed to linear flow of axial CF pumps to decrease suction at times of low flow and risk of left ventricular suck-down and ventricular arrhythmias. Further modifications in bearing design have been made to allow more streamlined function and to reduce complications of thrombosis, haemolysis, and device wear due to contactless bearings[3,23].

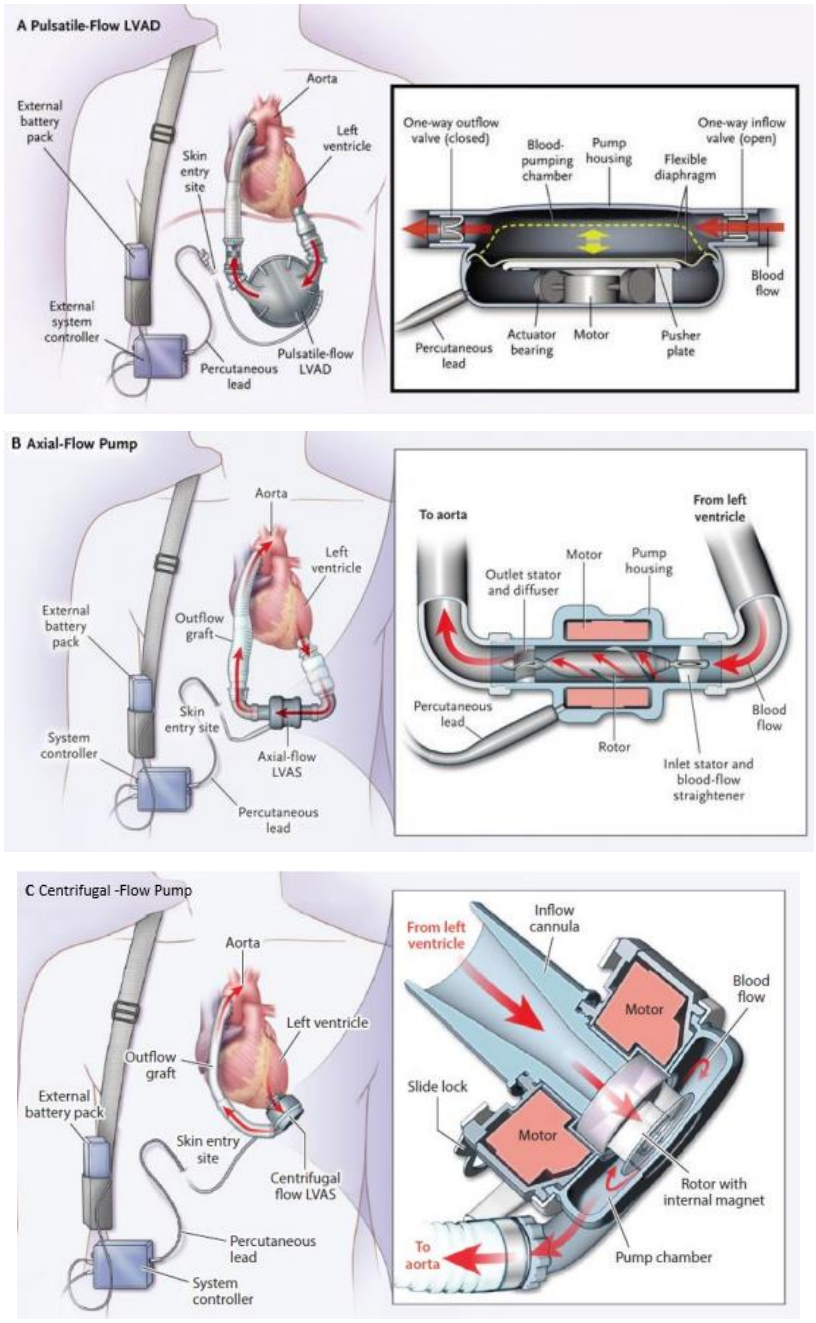


Figure 1.3 – Representation of different LVAD technologies. **(A)** PF devices; **(B)** CF axial pumps; **(C)** CF centrifugal pumps[4].

Overall, the second and third generation LVAD are smaller in size and require less surgical dissection and time for implantation. In addition, since they have fewer moving parts, these devices have greater durability with an expected lifespan of 5–10 years. Studies have also demonstrated the increasing evidence of preserved and even improved microcirculation and organ function with non-pulsatile flows in patients with end-stage heart failure, and the importance of continuous mechanical left ventricular unloading in reducing pulmonary

pressures. For these reasons, since 2008, CF-LVADs are the most used since they guarantee an improvement in the survival and quality of life of the patient, and in the clinical output (Figure 1.4)[3].

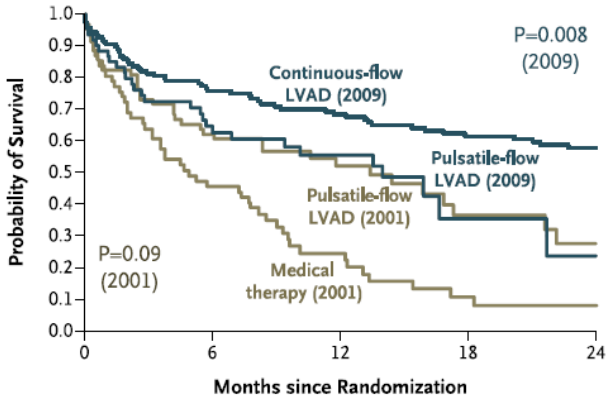


Figure 1.4 – Survival in end-stage heart failure as managed by optimum medical therapy, PF LVADs, and CF LVADs[3]. P obtained by log-rank test.

It can be observed how the advent of durable MCS devices, rather than optimum medical therapy, has drastically improved the survival of the patients already after six months from the implantation. In addition, the introduction of CF pumps has significantly gained a better probability of survival, >70% after one year, compared to PF pumps, <60% after one year.

1.1 Second-generation ventricular assist devices: Heart Mate II LVAD

The HeartMate II (HMII) was the first device to be CE mark authorised for BTT and DT in November 2005. FDA approval was obtained for implantation as a BTT in April 2008 and as DT in January 2010.

The device (Figure 1.5) is an axial flow rotary pump that contains a titanium-coated internal rotor (to reduce blood cell adhesion) with helical blades that curve around a central shaft. The only moving part of the pump is the rotor which is suspended with ceramic–aluminium oxide ball and cup bearings designed for low wear and long-term reliability. Vanes attached to the rotor help propel blood forward to achieve flows from 3 to 10 L/min. The pump has a weight of 281 g and a volume of 63 mL[16,22].

A  B 

Figure 1.5 – (**A**) The HeartMate II LVAD, (**B**) Implanted pump and system controller [25].

The device is connected to an external monitor that displays (Figure 1.6)[16]:

- Speed expressed in revolutions per minute (RPM). There are two modes to operate: fixed (set to an RPM between 6000 and 15000) and emergency "power-saver" mode (8000 RPM or the fixed speed if it is <8000). It can be modified only by qualified operators.
- Pump power in Watts, that is the amount required to power the motor and it is in the range of 0 to 25.5 W.
- Flow in L/min, it is estimated based on the pump speed and the power input and it can vary from 3 to 10 L/min. Except for very low or high pump speeds, the relationship between power and flow at given speed is almost linear.
- Pulsatility index (PI), it quantifies the variation in flow ("flow-pulse") due to the augmentation of the continuous flow, through the device, with each contraction of the native ventricle. PI is a dimensionless value from 1 to 10 and it is determined by the interaction among ventricular preload, myocardial contractility, and the amount of assistance provided by the device. It is defined as (eq. 1.1):

$$PI = \frac{Q_{max} - Q_{min}}{Q_{avg}} \times 10 \qquad \text{(eq. 1.1)}$$

where Q_{max} and Q_{min} are, respectively, the maximum and minimum pump flow rate and Q_{avg} is the medium flow rate of the pump. Higher PI occurs when ventricular filling is increased or the device is providing a low level of support, whereas a lower

PI corresponds to an underfilling of the ventricle or to a high level of support provided by the device. In clinical use, PI is in the range between 3 and 4.

Figure 1.6 – External monitor of the HMII[25].

In axial flow pumps (Figure 1.7), the flow direction is maintained along the axis of rotation. Inlet flow enters the pump along the axis of rotation of the impeller. The impeller initially directs blood along the axis of rotation but there is also an unavoidable slight spiral component to the flow. As blood exits the pump, this spiral flow component is usually redirected by an outlet stator to be in the axial direction to enhance pump efficiency[26].

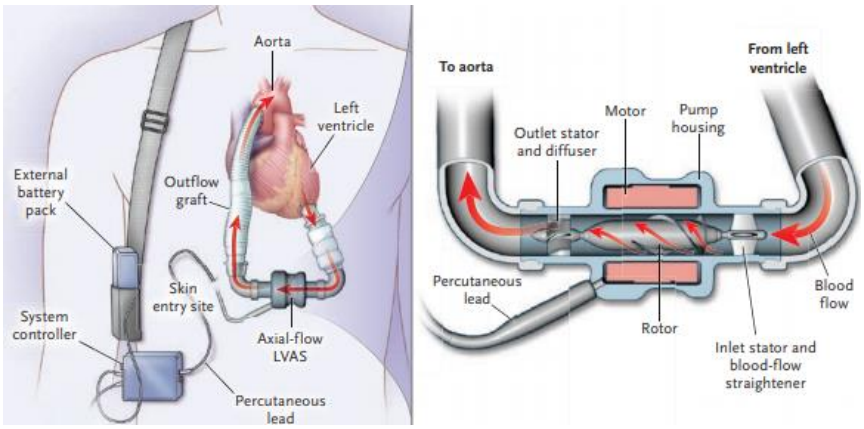


Figure 1.7 – Axial pump components[27].

1.2 Third-generation ventricular assist devices

Third generation devices such as the HeartMate III (HM3) and HeartWare HVAD operate via a hydro-magnetically levitated rotor without mechanical bearings and can deliver up to 10 L/min of flow. The absence of mechanical contact points within the pump eliminates friction and heat generation, which also improves device durability. In addition, advances in miniaturization allow for a minimally invasive surgical approach: the pump can be implanted in the pericardial space, thus simplifying operative placement by eliminating the need of a pump pocket[28].

Centrifugal pumps (Figure 1.8) accelerate blood in a circular manner causing it to generate centrifugal pressure at the periphery of the pump. Blood enters at the centre of the pump along the axis of rotation of the impeller. Thus, the direction of the exiting flow at the periphery of the pump is perpendicular and offset to the axis of rotation. This flow pattern is characteristic of all centrifugal pumps.

Compared to axial pumps, CF pumps are smaller and with a tubular configuration, thus they can be implanted faster and even less invasively, and therefore are probably more cost-effective.

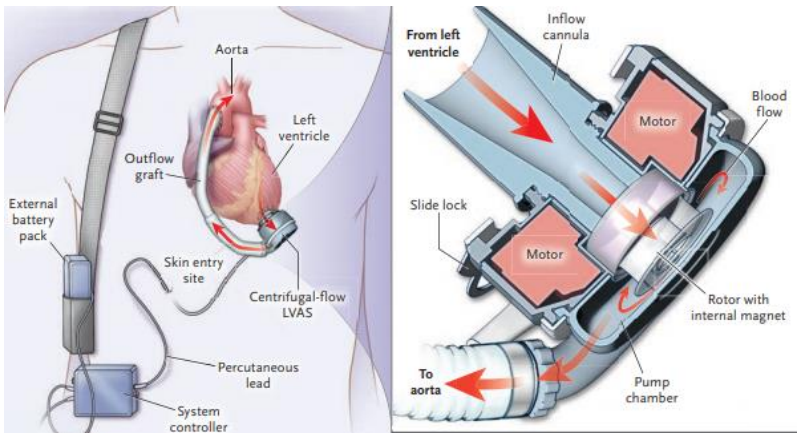


Figure 1.8 - Fully-magnetically levitated centrifugal flow pump components[27].

1.2.1 HeartWare HVAD

The HeartWare Ventricular Assist Device (HVAD; HeartWare Inc., Framingham, MA USA) is a small CF LVAD with a centrifugal impeller approved by the FDA as BTT in 2012[4].

The pump is surgically placed within the pericardial space with the integrated inflow cannula positioned in the left ventricle, avoiding abdominal pump placement. The pump housing is made of titanium and a robust organic thermoplastic called 'polyether ether ketone (PEEK)' for improved durability and strength. The only moving part is the impeller, suspended by magnetic forces created by large permanent rare-earth magnets placed strategically to contribute to the internal suspension system and provide radial support to the impeller. These magnets push the impeller towards the inflow tract, creating a passive magnetic force. This action is counterbalanced by hydrodynamic forces created by the blood when the pump is switched on, thus the blood enters the flow channels of the impeller creating a completely frictionless and almost noiseless system[27]. In particular, the net effect of impeller rotation is increased blood pressure and flow from the inlet to the outlet. The energy necessary to rotate the impeller is provided through electromagnetic coupling between permanent motor magnets enclosed within the impeller and the motor stators located in pump housings. Each motor stator consists of six copper wire coils that are sequentially charged by electrical current, creating an electromagnetic force that rotates the impeller. These electromagnets have the effect of dragging the motor magnets (and impeller) around an axis of rotation at an adjustable speed. The amount of blood flow through the pump at a constant impeller rotational speed is determined by the differential pressure across the pump. The difference between preload (left ventricular pressure) and afterload (aortic pressure) is the most important hemodynamic parameter affecting blood flow through the pump[29].

In order to characterize global and local conditions within the pump, computational fluid dynamics (CFD) analysis was performed and demonstrated three flow paths (Figure 1.9)[29]:

- The primary flow path is through the inflow cannula and into four impeller flow channels after which blood is collected in the housing and exits the pump through the outflow graft

- The secondary flow path starts under the impeller and goes upward through the annular gap between the impeller and the center-post and then re-enters the primary flow path
- The tertiary flow path starts at the impeller slots and leads to the hydrodynamic thrust bearings at the top of impeller where blood re-enters the primary flow path and exits the pump outlet.

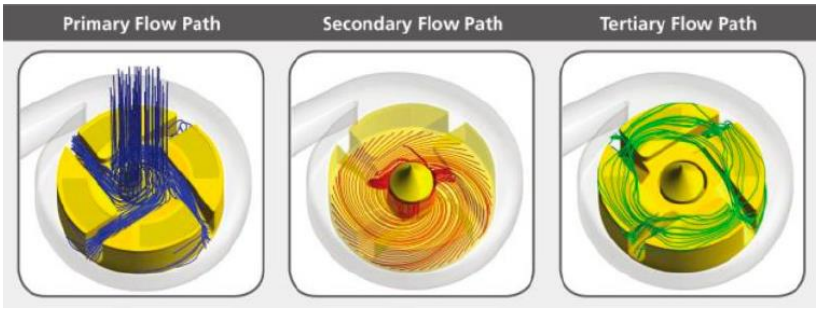
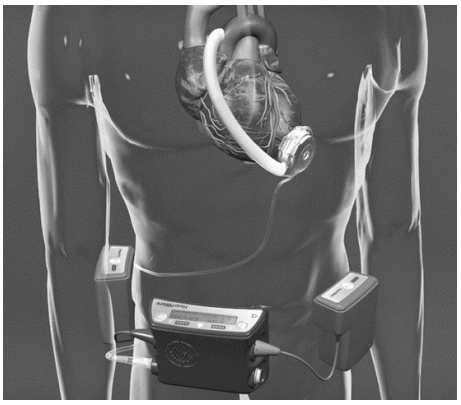


Figure 1.9 – CFD analysis used to characterize pump flow paths.

The pump is connected to external system components (Figure 1.10 A and B) by a driveline that exits the patient's abdominal wall. A controller operates the pump, regulates power, monitors system performance, and displays alarm notifications. The pump has a displaced volume of 50 mL and weighs 160 g[27].

A



B

Figure 1.10 – (**A**) The HeartWare HVAD device system, (**B**) Implanted pump with system controller[29].

The system can be powered by a pair of rechargeable direct-current lithium-ion batteries, alternating-current power from an electric wall outlet, or a 12-V direct-current power source. A monitor displaying pump performance (Figure 1.11) is used to set and adjust the operating

parameters, and it provides a means to download data from the controller[30]. In particular, the operating parameters of HVAD are[26]:

- Pump flow: up to 10 L/min
- Pump speed: the range is 1800-4000 RPM but in the clinical use the operating range is between 2300 and 3200 RPM
- Pump power: the power consumption is between 3-6 W

Figure 1.11 – Monitor display of the HVAD[29].

1.2.2 HeartMate 3

The HeartMate 3 (HM3) LVAD is a CF centrifugal pump designed by Thoratec Corp., which was acquired by St. Jude Medical Inc. (Pleasanton, CA, USA) and later by Abbott (North Chicago, IL, USA), to supply hemodynamic support in left ventricular heart failure patients, either as a bridge to transplant (BTT), myocardial recovery, or as destination therapy (DT)[31].

The HM3 (Figure 1.12) is surgically positioned in the pericardial space, with an inflow cannula inserted into the left ventricular apex and an outflow graft anastomosed to the ascending aorta. The implanted components include the inflow cannula, pump housing, motor, control electronics, outflow graft and bend relief, and percutaneous driveline. All of the wetted surfaces of the pump are in titanium, except for the woven polyester grafts and the polytetrafluoroethylene (PTFE) washers at joints. The textured titanium surface promotes the development of a stable, adherent pseudoneointima immediately upon contact

with blood, thereby insulating subsequent blood flow from contact with nonbiologic materials and reducing the risk of thromboembolism. The rotor instead is fabricated with smooth, polished surfaces. The device has a weight of 474 g and a volume of 168 mL[32].

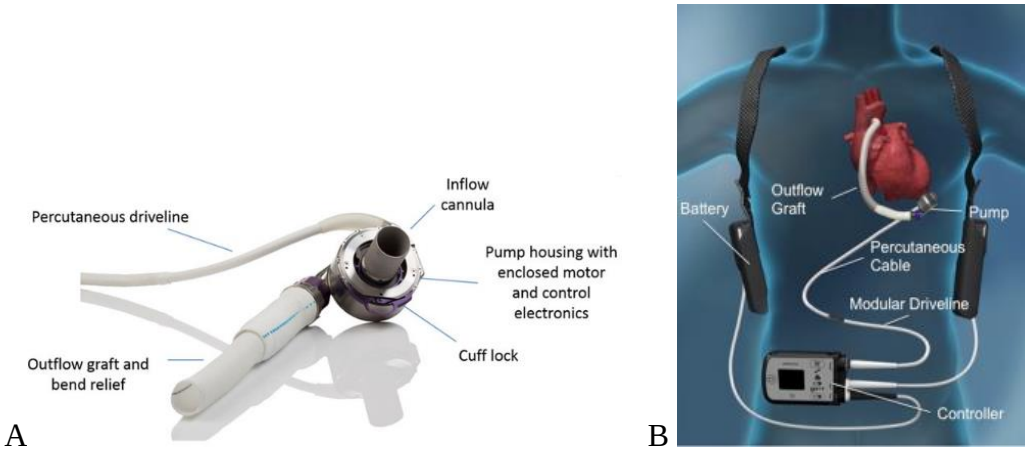


Figure 1.12 –The HeartMate 3 pump (**A**) and its components (**B**)[33].

Except for the system controller, all external components are identical for both the HeartMate II and HeartMate 3. The fundamental strategy for the design of the HeartMate 3 was to adopt the aspects of mechanical circulatory support technology that have worked well (*i*.e., the reliability and small size of a rotary pump), but also to pursue a different technological path that addresses the factors implicated in the most significant adverse events. These innovations involve[34]:

- A fully magnetically levitated rotor (Figure 1.13) (Full MagLev, St. Jude Medical, Inc., Pleasanton, CA) based on the use of a magnetic bearing, an alternative approach to hydrodynamic bearings to achieve rotor levitation without imposing high shear and compressive forces on blood

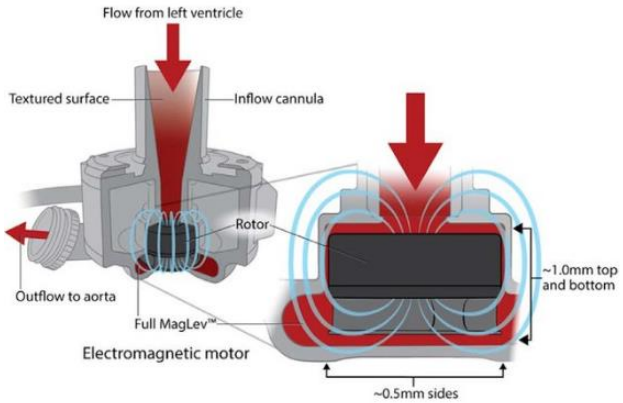


Figure 1.13 - Schematic working principle of the HeartMate 3[35].

- Wide blood-flow passages to minimize red blood cells (RBCs) trauma
- Intrinsic pulsatility (artificial pulse) that washes the pump preventing the formation of microthrombi

These improvements in technology have been shown to improve hemocompatibility and so they may mitigate bleeding by allowing for changes in anticoagulation strategy. Moreover, compared to HMII, the device shows the expected inverse relationship between the pressure head across the pump and flow through the pump with the general convention that the slope of this relationship is steeper for the axial-flow HMII than for the centrifugal HM3 (Figure 1.14). Thus, in the typical ranges of clinical operation, a change in pressure head across the pump results in a greater change in flow for the HMII than for the HM3[36].

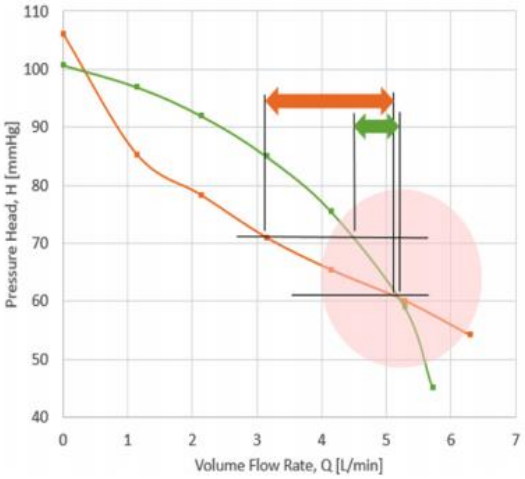


Figure 1.14 – Y-axis: pressure head H, X-axis: volume flow rate Q. The orange circle covers the clinical usage range. HeartMate II typical speed is 9,000 rpm (orange curve), and HeartMate 3 typical speed is 5,400 rpm (green curve). Orange arrow: change in flow rate for HMII, green arrow: change in flow rate for HM3[36].

Another important feature in HM3 technology is related to the volumetric shear stress distribution and values that act on the blood flow. Computational fluid dynamics analysis on shear stress acting inside HM3 pump was performed and compared with the corresponding values for HMII. The nominal operating condition was 5.4 L/min and 65 mmHg pump head[33].

Shear stress distribution (Figure 1.15) is relatively homogenous and smooth across the flow passages for both pump types. Typical high shear areas for the axial impeller were confined

at the blade tip, whereas HM3 impeller blades exhibit shear stress at the concave side of the blade leading edge and the convex side of the blade trailing edge.

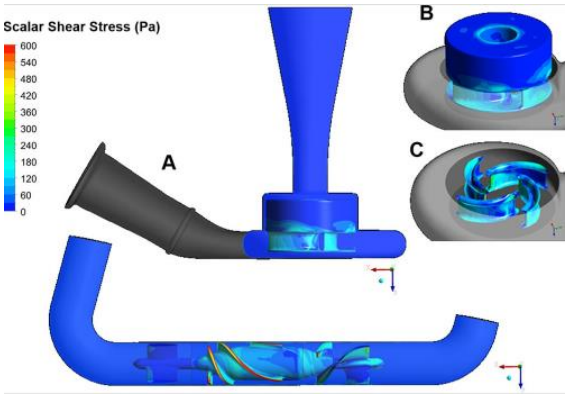


Figure 1.15 – Scalar shear stress surface contour plots at 5.4 L/min and 65 mmHg of HM3 and HMII (A), HM3 rotor well (B), and HM3 rotor blades (C)[33].

Moreover, the shear stress histogram (Figure 1.16) for the nominal operating condition indicates that more than 99% of the blood volume inside HM3 is exposed to low shear stresses (<50 Pa), compared with 97% of pump volume in HMII. Less than 0.03% (3.3 mm^3) of HM3 is exposed to high shear values >150 Pa, compared with 0.09% (11 mm^3) of HMII. The surface area with wall shear stress exceeding 150 Pa was 7.7% (134 mm^2) of the impeller in HM3, compared with 26% (604 mm^2) in HMII.

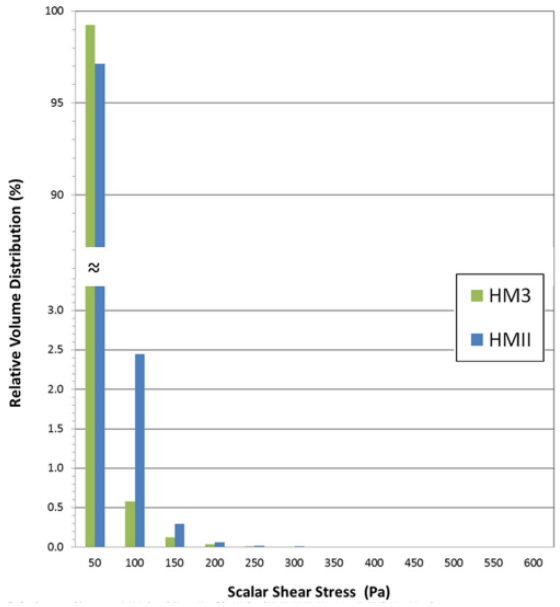


Figure 1.16 – Scalar shear stress histogram at nominal conditions (5.4 L/min and 65 mmHg pump head) for HMII and HM3[33].

As shown for the other LVADs, on the external controller are indicated the parameters of the pump:

- Pump flow: up to 10 L/min
- Pump speed: the range is from 3,000 to 9,000 RPM but the clinical use is from 4800 to 6500 RPM. The artificial pulse mode (Figure 1.17) is always used, which varies the rotor speed from the user set speed every 2 seconds to produce changes in blood flow and arterial blood pressure. It leads to a reduction of stagnation regions in the left ventricle[33].

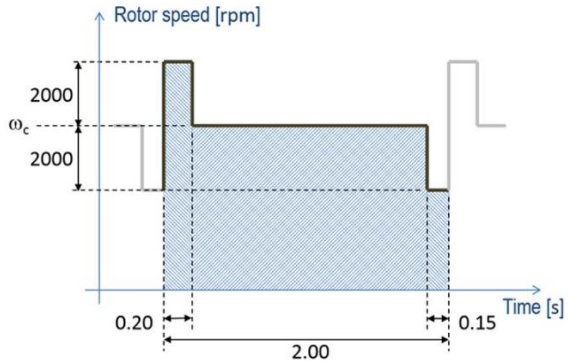


Figure 1.17 – Artificial Pulse Mode: every 2 seconds, the rotor speed decreases by 2000 RPM for 0.15 s, then increases by 4000 RPM for 0.2 s and then returns at the set value[33].

- Pump power: direct measure of the tension and the current related to the motor of the pump
- Pulsatility Index: indication of cardiac pulsatility and it varies from 1 to 10

The following Table 1.4 summarizes properties and specifications of the continuous flow LVADs described.

Device Type	HeartWare HVAD System	HeartMate II	HeartMate III
Speed range, rotations per minute	2400–3200	6000–15,000	3000–9000
Rotor design	Centrifugal	Axial	Centrifugal
Pump position	Intrapericardial	Pump pocket	Intrapericardial
Blood flow gaps, mm	≈0.05	≈0.08	≈0.12
Food and Drug Administration–approved indication	Bridge to transplant (2012) Destination therapy (2017)	Bridge to transplant (2008) Destination therapy (2010)	Bridge to transplant (2017) Destination therapy (2017)
Magnetic levitation	*		*
Artificial pulsatility			*
High inlet suction		*	

Table 1.4 – Device types currently approved by the US Food and Drug Administrations and their mechanical properties. * Notes the presence of the characteristic[37].

1.3 Post implant complications in ventricular assist device

Left ventricular assist devices (LVADs) are a reliable therapeutic option for the treatment of patients with advanced and end-stage heart failure. Modern LVADs are miniaturized, electromechanical, fully implantable, continuous flow pumps that work in parallel with the heart to decrease the loading of patient's left ventricle while increasing the cardiac output. As shown previously, these devices restore hemodynamic and functional capacity to improve patient survival. Nowadays, LVADs are approved as BTT and as long-term DT therapy[38].

Despite the hemodynamic efficiency, the improvement in design, and clinical management of these devices, the main challenge is to minimize the high rate of adverse events that occur in the long-term outcome. According to the eight annual INTERMACS report[6], 60% of the patients are re-hospitalized at least once after six months of device implantation. At 1 year after the implant, the rate of hospitalization has been estimated as high as 65% to 80%, for both axial and centrifugal-flow devices. As expected, the state of cardiogenic shock (INTERMACS 1) at time of implant has been correlated with higher risk of adverse events. However, even among healthier patients (INTERMACS 4-7) there is high morbidity at 1 year follow-up with 80% readmission rate (Figure 1.18)[37].

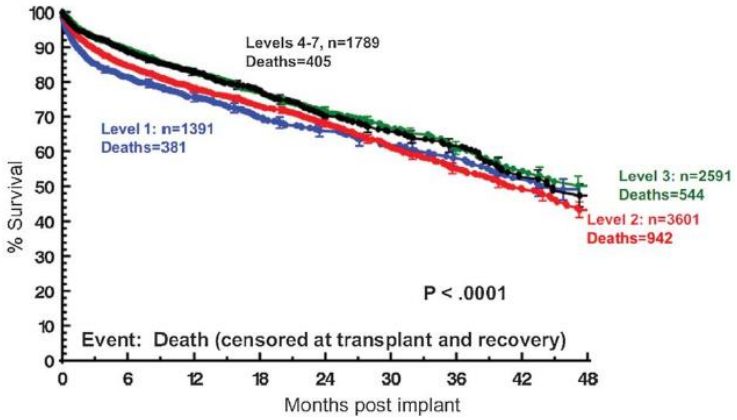


Figure 1.18 – Kaplan Meier survival curves, stratified by INTERMACS profile at time of implant[4].

Preexisting states of illness, organ dysfunction, complex physiology due to anticoagulation therapy predispose VAD patients to a high risk of complications that may lead to the death of the patient (Figure 1.19). These include bleeding, infections, right heart failure (RHF), pump thrombosis (PT), stroke, and device malfunction. Consequently, concomitants risks of PT, stroke, and bleeding represent the complex hematologic challenge in MCS, expressing as increased bleeding risk and hypercoagulability.

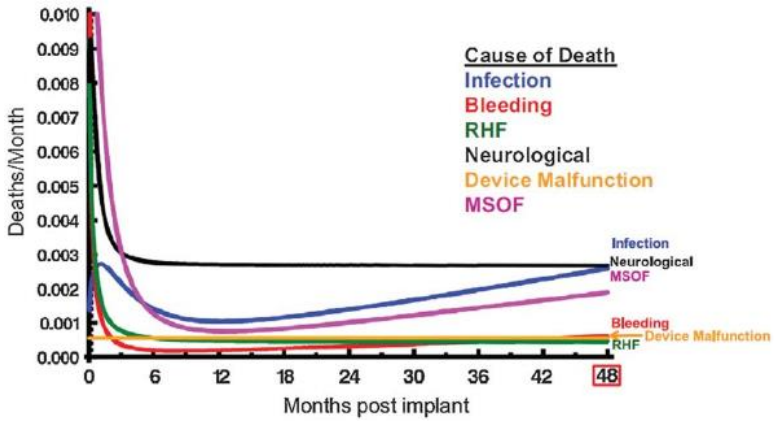


Figure 1.19 – Instantaneous risk (hazard curves) of death after primary implantation of CF LVAD, stratified by specific cause of death[4]. MSOF, Multiple System Organ Failure.

1.3.1 Hemocompatibility-related adverse events: bleeding and thrombosis

Continuous flow LVADs have replaced pulsatile devices with an improvement in overall outcomes. However, the incidence of gastrointestinal (GI) bleeding has increased

significantly and hemorrhage remains major concern in patients receiving LVAD for end stage heart failure, resulting in rise of hospital admissions, procedure burden, and cost. Nowadays, bleeding, including surgical, gastrointestinal (GI), and other bleeding, is the most common and critical adverse event, reported to occur from 18% to 23% of the LVAD patients with a peak in the first 3 months after implantation[39].

Crow et al.[40] demonstrated that the incidence of GI bleeding has been quantified to be 6.8 events/100 patient years (10%) in pulsatile LVADs while in CF LVADs it has been estimated at 63 events/100 patient years (20 to 30%), as shown in Figure 1.20.

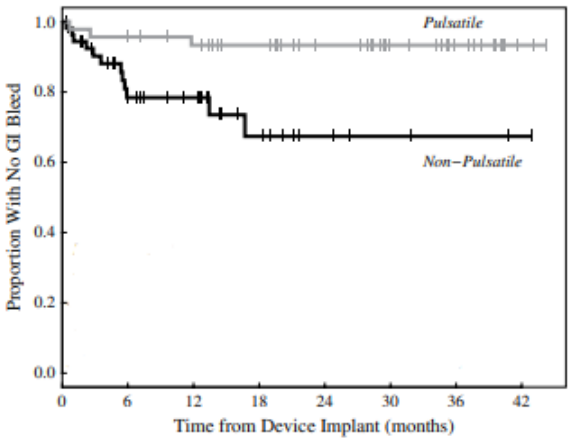


Figure 1.20 – Gastrointestinal bleeding events over time after LVAD implantation. Comparison between CF LVAD and PF LVAD.

From a clinical point of view, the risk of bleeding in CF LVAD is multifactorial:

- Exogenous factors: anticoagulation and antiplatelet therapy
- Endogenous causes: fibrinolysis
- Intrinsic properties of the device: the effect of the LVAD on endothelium, platelets, von Willebrand Factor (vWF), and angiogenesis
- Pre-disposing systemic conditions in the patients requiring such devices (hepatic and renal dysfunction).

Even if the anticoagulation therapy, after LVAD implantation, influences the occurrence of GI bleeding, an important feature is related to the altered hemodynamics that occurs in CF LVADs. In fact, the physiological pulsatility found in the normal cardiac cycle is lost and substituted with a narrowed arterial pulse pressure with a decrease aortic valve opening. This clinical setting mimics what happens in aortic stenosis, which is related to Heyde's

syndrome, characterized by the association between bleeding gastrointestinal angiodysplasias and aortic stenosis[41].

Additional mechanisms that may contribute to bleeding are the acquired von Willebrand syndrome (avWs) and alterations in platelet functions due to the high shear stress imposed by the pump. In fact, the shear stress of LVAD device can easily degrade and cut the high molecular weight (HMW) multimers of the von Willebrand factor into smaller, medium and low molecular weight fragments, which harms thrombosis, interferes with platelet aggregation, and leads to bleeding. At the same time, abnormal platelet aggregation is partially caused by the interactions with artificial material and turbulent blood flow through the LVAD. Platelets tend to exhibit an increased sensitivity to shear stress, lysing at velocities far below speeds that would affect erythrocytes[42].

Even if less frequent than bleeding, thrombosis is a severe complication of both short and long-term use of LVADs and occurs in 2–13% of patients with a continuous-flow LVAD (axial-flow 4–13%, centrifugal-flow 8%)[43]. It can cause both the onset of cerebrovascular events (thromboembolic stroke) and the obstruction of the device (pump thrombosis). Pump thrombosis is related to the development of clot within the flow path of any component of the pump, including the titanium inflow cannula, the rotor and the outflow graft. Thrombus can originate in the pump or travel from the left atrium or left ventricle, and set in the pump components and it can lead to thromboembolic stroke, peripheral thromboembolism, LVAD malfunction with reduced systemic flows, LVAD failure with life-threatening hemodynamic impairment, cardiogenic shock, and death.

The development of the thrombotic complications depends on:

- Interaction of blood with the biomaterial of the pump which activates blood components through contact mechanisms
- Hemolysis - and the following stimulation of the coagulative response - of erythrocytes
- Fluid dynamics of the device which is characterized by a non-physiologic flow (continuous flow instead of pulsatile), the presence of turbulences in the blood stream, and the high shear stress imposed by the pump to the blood components.

The occurrence of this events varies with the type of device implanted. *Starling et al*.[44] found rates of pump thrombosis in patients with HMII raised of 6.2% over 2 years, from 2.2% at 3 months post-implantation in 2011 to 8.4% in 2013. Another study conducted by INTERMACS reviewed data from 6251 HeartMate II LVAD implantations, considering a period from 2008 through 2012, and found an increase of pump thrombosis rates by 3% at 6 months post-implantation, when comparing data from the intervals 2008-2011 to 2011-2013[45]. Data from *Najjar et al*.[46] and the ADVANCE trial investigators showed similar rates of pump thrombosis in HVAD patients, with 4% of patients experiencing pump thrombosis at 6 months post-implantation.

A study conducted by *Florisson et al.*[47] analyzed publications related to MOMENTUM 3 randomized control trial (RCT) in which there is the comparison between patients who received HeartMate 3 (HM3) and patients who received HeartMate II (HMII). The results showed that HM3 had better outcomes compared to HMII with a lower rate of pump thrombosis (1.1% vs 15.7%), stroke events (10.1% vs 19.2%) and ischemic stroke (6.3% vs 13.4%) at 2-year follow-up while markers of quality of life and functional capacity were comparable between the 2 devices at 6 months.

The same study reported also the overall results derived from ENDURANCE RCT, a multicenter and prospective evaluation, which compared patients with HMII to patients with HVAD. Outcomes from retrospective studies comparing HMII with HVAD varied, with some publications reporting higher rates of right ventricular assist device use (29% vs 15%), gastrointestinal bleeding (30% vs 0%), cerebrovascular accident (44% vs 10%), transient ischemic attack (5% vs 2%) and higher cumulative risk of infection and hemorrhagic cerebrovascular accident with HVAD. Other publications[48,49,50], instead, showed an heterogeneity in the data and no strong differences in any outcomes regarding the 2 groups.

In conclusion, patients with centrifugal flow HM3 show better outcomes than those with axial flow HMII. Even if there is some variability in the outcomes of retrospective studies, patients with centrifugal flow HeartWare HVAD have similar outcomes to those with axial flow HMII, especially when there is a strict blood pressure control after implantation that allows a reduction of stroke rates in HVAD subjects.

1.3.2 At the basis of adverse events: platelets, Von Willebrand factor, and pharmacological therapy

This section focuses the attention on the role of three factors in the risk of the development of complications in LVAD patients:

i. platelets;
ii. vWF;
iii. anticoagulation therapy.

For the first two factors, a brief review of their biological function and the following alterations in the action mechanisms, as consequence of the presence of the device, are described. Afterwards, a description of the pharmacological therapy and its interaction with the organism is shown.

Platelets

Platelets, or thrombocytes, are anucleate blood cells of 2-3 μm, derived from the cytoplasm of megakaryocytes, and play a critical role in hemostasis. In correspondence of the site of vascular injury, the matrix proteins (von Willebrand factor and collagen) of subendothelial layer are exposed to blood. The exposed vWf and collagen activate the platelet adhesion receptors glycoprotein Ibα (GPIbα), which belongs to the GPIb-IX-V complex with VWf binding, and glycoprotein VI (GPVI), which is a receptor for collagen binding. The activation of the two pathways triggers an intracellular signaling cascades leading to final platelet aggregation forming a primary hemostatic plug to stop bleeding.

Together with this receptor-based mechanism, different studies[51,52,53,54] have demonstrated the role of shear stress in platelet activation (PA). It is a non-linear phenomenon in which the value of platelet trauma increases as an exponential function depending on shear rate and time. This shear-induced mechanical activation is consequently related to thrombosis through two stages: (i) exposure to supraphysiological shear stresses and abnormal flow pattern during the passage in the pump, and (ii) in thrombus formation which involves both platelet aggregation and fibrin clot generation.

In order to guarantee physiological flow rates (5-6 L/min), LVADs work at high rotation speed of the impeller (axial pumps: 6000-15000 RPM, centrifugal pumps: 2000-4000 RPM) which generates high level of shear rate, defined as follow (eq. 1.2):

$$\gamma = \frac{dv}{dr} \qquad \text{(eq. 1.2)}$$

where γ is the shear rate [s^{-1}], perpendicular to the direction of the fluid motion, v is the velocity of the blood and r is the radius of the channel considered. According to Newton's law, shear stress is defined as (eq. 1.3):

$$\tau = \eta \times \gamma \qquad \text{(eq. 1.3)}$$

where τ is the shear stress [Pa] and η is blood viscosity [Pa × s].

In physiological condition, vascular flow is characterized by low wall and fluid shear stresses, ranging from 0.1 to 1 Pa in venous flow to 5 Pa in arterial flow, reaching a peak at 6 Pa in the arterioles. When the blood flows inside LVAD pump, it exposes platelets to higher shear stresses, ranging between 400-500 Pa, and so they act as agonist for PA. Moreover, even if the exposure time of the platelets to these levels of shear stress is very short (< 1 s), it is enough to activate and sensitize the cells: in fact they are not able to return in their quiescent condition but remain in an pro-active state, in which they are activated even by levels of shear stress that are far below the ones imposed by the device. Another important feature is that platelets are able to "memorize" the level of shear stress imposed at every cycle of the pump, thus the level of PA is higher after every passage in the pump[55]. As a consequence, platelet dysfunction will affect normal hemostasis leading to thrombosis and bleeding.

Von Willebrand Factor

Von Willebrand factor is an adhesive multimeric glycoprotein synthesized by endothelial cells and megakaryocytes. Its main functions[56] are:

- Promoting platelet adhesion and aggregation at the site of vascular lesion through the interaction with the exposed extra-cellular matrix (ECM) collagen and the receptors present on the platelet membrane. The binding with platelet receptors depends on the shear rates that facilitate subendothelial surface-platelet interaction.

In the condition of high shear rate, the role of vWf is crucial: it passes from a globular structure to a straight chain to expose a higher number of intracellular domains, increasing biological activity.

- Protecting the coagulation factor VIII (FVIII) against proteolytic activity of C-reactive Protein (CRP) system by forming a vWf-FVIII complex in the circulation.

The mature glycoprotein is a linear homopolymer of subunits bonded together through disulfide bonds between C- and N- terminals of the monomers. The multimer is the result of a series of intracellular events that brings to the storage and/or secretion of a heterogeneous group of multimers of different sizes, properly called vWf. The storage of these multimers can be found in Weibel-Palade bodies, endothelial cell vesicles that release their content ("ultra-large" multimers) through the membrane by means of a regulated system, and in the platelet's α-granules. In the hematic stream, these high molecular weight (HMW) multimers interact with the protein ADAMTS-13 that regulates their dimension operating a cleavage.

Each protein domain needs to remain intact so that the mature vWf can preserve its functions. Mutations related to a single domain can bring instead to a deficit and /or an altered function of the factor, causing the so called Von Willebrand disease (VWD). The vWf activity is related to the presence of HMW multimers and, as consequence, their reduction implies a deficiency of the factor, more or less severe depending on their concentration in the blood flow.

Literature[11,57,12,58] describes clearly how the high shear stress generated by the LVAD continuous flow pumps determine a progressive and irreversible degradation of vWf. This phenomenon - known as acquired Von Willebrand syndrome (avWS) – is a risk factor for bleeding. As mentioned before, the supraphysiological shear stress imposed by LVAD devices acts on the weaker disulfide bonds of vWf multimers, tearing apart vWf monomers. In parallel, it leads to enzymatic degradation cleavage of large, active vWf multimers into small, nonactive vWf fragments by vWf protease ADAMTS-13. As a result, an acquired vWf deficiency develops, which significantly affects sprouting angiogenesis since vWf drives the formation of Weibel-Palade bodies that stores regulators of angiogenesis and inflammation. Thus, the dysregulation of these regulators may lead to endothelial cells proliferation and migration to vascular endothelial growth factor- dependent pathways, with increased neo-angiogenesis and vascularization.

Pharmacological therapy

To prevent thrombosis, one of the major risks of post-implant complications, a daily therapy of antiplatelet agents and anticoagulants is administered to CF LVAD recipients.

Antiplatelet agents inhibit platelet aggregation and the following formation of the clot to prevent thrombus formation.

Anticoagulants, instead, inhibit the synthesis and the function of some coagulation factors, therefore they act directly on the coagulation cascade.

The typical pharmacological drugs used are, respectively, Aspirin (acetylsalicylic acid, ASA) and warfarin.

Before describing the action of these drug, a brief description of the physiology of the hemostasis is given. It consists in a series of coagulation processes by which vascular damage is sealed up to limit blood loss following an injury. It can be divided in four phases[59]:

i. Vascular, contraction of the injured vessel mediated by smooth muscle cells, vasomotor reflex of autonomic nervous system, and release of vasoconstrictors (endothelin)
ii. Platelet, divided in the following processes: (i) adhesion and activation – integrins on platelet membrane bind to collagen, proteoglycans and glycoproteins of subendothelial layer of the vessel, and binding of GPIb platelet receptor to vWf, (ii) changing in shape – adhesion of platelets to sub endothelium triggers a signal cascade that leads to the formation of pseudopods on platelet surface to allow platelets aggregation, (iii) granules secretion – to amplify hemostatic response, (iv) aggregation.
   Parallel to platelets, there is the activation of the following enzymatic systems:
   - Phospholipase A_2: it is activated by ADP and releases arachidonic acid from phospholipids present on the membrane. This fatty acid is the precursor of thromboxane A_2 (TXA_2) which further amplifies platelet aggregation.
   - Phospholipase C: it is activated by subendothelial collagen.
iii. Coagulation, formation of insoluble fibrin clot after the activation of the fibrinogen (fibrin precursor). Coagulation factors (numbered from I to XIII, all present in the blood except for FIII; factor I is fibrin) involved in this process are synthesized by hepatocytes and released in the blood flow as inactive form. For the synthesis of factors II, VII, IX, and X is necessary vitamin K, cofactor for their carboxylation. The enzymatic cascade

(Figure 1.21) that characterizes this phase can be triggered by two pathways, known as tissue factor pathway (extrinsic) and contact pathway (intrinsic), which converge in the final common pathway. They principally differ for the activating initial agent and the number of factors involved in the cascade.

- Intrinsic pathway: triggered by the contact of the blood with negative charged surfaces, in particular the factor XII is converted in the active form XIIa when blood interacts with the exposed collagen of the vessel. It is slower in time than extrinsic pathway and involves more factors.
- Extrinsic pathway: triggered by the interaction between blood and the tissue factor (TF or FIII) expressed by fibroblasts of sub-endothelial matrices and secreted by injured endothelial cells.
- Common pathway: FX is activated and interacting with FV and calcium ions forms a prothrombinase complex that converts FII (prothrombin) in FIIa (thrombin). Thrombin in turn catalyzes the conversion of the fibrinogen in fibrin monomers and the polymerization of these monomers allow the clot formation.

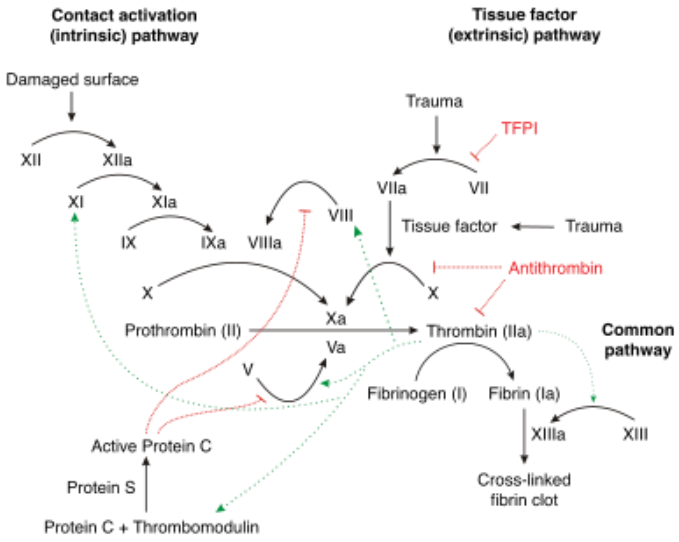


Figure 1.21– Coagulation cascade with the effects of positive feedback (green lines) and inhibition factors (red lines).

iv. Fibrinolysis, enzymatic breakdown of fibrin blood clot. It is operated by plasmin that cuts the clot in various places, leading to the production of fragments that reach the circulation and are cleared by other proteases.

It is now possible to show the action mechanisms of antiplatelet agents and anticoagulants[60]:

- Aspirin: it negatively interacts with platelet aggregation function, preventing the activity of cyclooxygenase (COX) enzyme which is responsible for the conversion of arachidonic acid in TXA_2 that, as explained above, plays an important role in platelet aggregation (Figure 1.22). However, there are two types of COX: COX-1 and COX-2. Both are switched off by acetylsalicylic acid through the acetylation of a serine residue localized near the active site of the enzyme. If COX-1, platelet dependent, is permanently inhibited, since platelets (anucleate) are not able to synthesize new messengers for TXA_2 synthesis, COX-2, present in nucleate endothelial cells, can be neo-synthesized by the cells themselves and then allowing the production of prostacyclin, inhibitor of platelet aggregation. For these reasons, an intrinsic limitation of the mechanism of Aspirin is, in specific conditions as a wrong dosage of the drug, relative to the inhibition of production by vascular endothelial cells of the antiaggregating prostaglandin, prostacyclin, leading to an higher risk of bleeding.

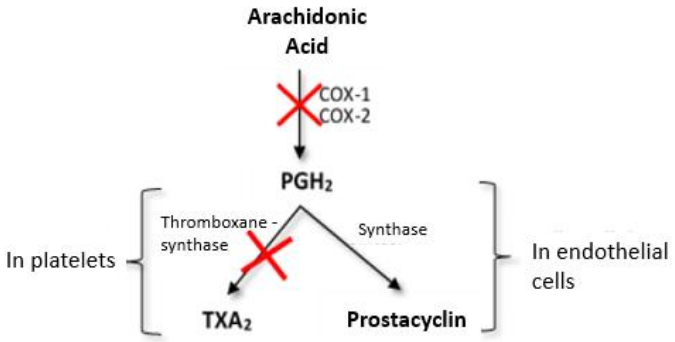


Figure 1.22 – COX-1 and COX-2 enzymes convert arachidonic acid in prostaglandin H_2 (PGH_2). PGH_2 is then converted in TXA_2 by thromboxane-synthase and, in parallel in endothelial cells by a specific synthase, in prostacyclin. Red crosses indicate the action of ASA.

- Warfarin: the anticoagulant property depends on its antagonist action on vitamin-K that is a cofactor of vitamin K epoxide reductase, the enzyme responsible for the activation of the coagulative cascade (Figure 1.23).

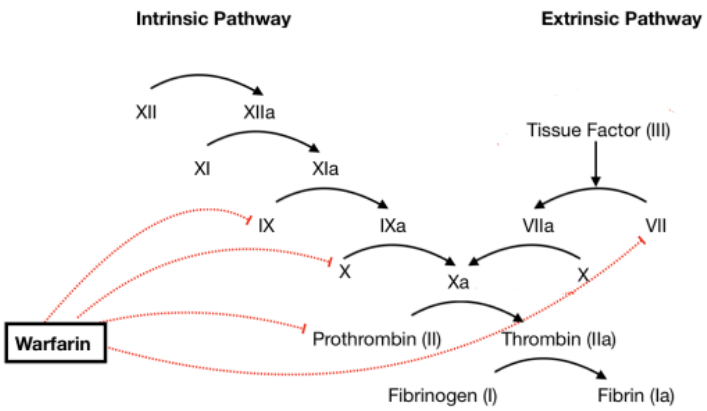


Figure 1.23 – Mechanism of action of Warfarin on coagulative cascade.

Although the administration of the pharmacological therapy is necessary for CF LVAD patients, the thromboembolic risk remains due to the possibility of the patient to develop a drug resistance. Moreover, the "standard" protocol for the dosage of the drugs does not consider the patient-specific response to the therapy. In this way the therapy could act negatively on the already delicate hemostatic balance and could not be appropriated for specific devices or patients. An upper- or lower-dosage of antithrombotic agents can determine a higher risk, respectively, of thrombosis or bleeding.

Chapter 2

Characterization of the factors responsible for LVAD hemocompatibility-related adverse events

Mechanical circulatory support devices, as LVADs, remain a valuable solution for patients with advanced and end-stage heart failure, resulting in a significant improvement in patient survival and quality of life. However, VAD therapy is plagued by post-implant adverse events including thrombosis and bleeding which, as shown previously, are strongly related to the following factors: platelet activation, vWf degradation, and antithrombotic pharmacological therapy.

As a consequence, this chapter focuses on the clinical practice used to evaluate the coagulation state of VAD patients during the post-implant, then – for each factor - on laboratory tests and clinical analysis which investigate the functionality of the factors with the aim to optimize the thrombogenic performance of the pump and the clinical outcome of the patients.

2.1 Clinical parameters

Clinical practice relies on a series of blood parameters and coagulation markers for the evaluation of coagulation capability, pharmacological response, and the consequent thrombotic and hemorrhagic risks in LVAD patients. The following Table 2.1 summarizes the most significative and utilized tests in clinical routine.

Blood Parameters	Acronym	Unit of measure	Normal range
Hematocrit	Hct	%	M = 40-54 F = 36-48
Hemoglobin	Hb	g/dL	M = 14-18 F = 12-15
Platelets	Plt	10^9/L	140-450
Plasma Protein			
Lactate Dehydrogenase	LDH	U/L	100-190
Fibrinogen	Fg	mg/dL	160-450
D-Dimer Fibrin	DD	μg/mL	< 0.5
Free Hemoglobin	fHb	mg/dL	< 5
Coagulation Markers			
Antithrombin	AT-III	%	80-120
Protrhombin Time	PT	sec	12- 14
Partial Thromboplastin Time	PTT	sec/ratio	19-30
International Normalized Ratio	INR	adimensional	0.8-1.2

Table 2.1 – Clinical parameters for hemostasis evaluation.

A complete blood count (CBC) is a set of medical laboratory tests that measures blood components and features. It indicates the amount of white blood cells, red blood cells, and platelets, the concentration of hemoglobin (Hb), and the hematocrit (Hct).

Hematocrit (Hct) measures the volume of red blood cells compared to the total blood volume (red blood cells and plasma). It is expressed as a volume percentage and the normal value for men is 40 to 54%, for women 36 to 48%. In LVAD patients, Hct values lower than reference range can indicate a damage to the erythrocytes induced by the pump, thus leading to hemolysis.

Hemoglobin (Hb) is the protein contained in red blood cells that is responsible for delivery of oxygen to the tissues. To ensure adequate tissue oxygenation, a sufficient hemoglobin level must be maintained. The normal Hb level for males is 14 to 18 g/dL, for females is 12 to 16 g/dL. In presence of hemolysis, the hemoglobin contained in the erythrocytes is released into the plasma, becoming free hemoglobin (fHb), with a normal range of 0-10 mg/mL. This parameter is an important index for the detection of the hemolysis induced by

VAD, in fact INTERMACS identifies values of fHb higher than 40 mg/mL as evidence of a significative hemolysis and a possible pump thrombosis[61,62]. This phenomenon is related to the pro-coagulative effect of red blood cell fragments which can cause the occurrence of a thrombus. Conversely, when the hemoglobin level is low, the patient has anemia which could be related to an hemorrhage caused by the device.

Platelets (Plt) are anucleate cellular fragments present in blood and they are considered an extremely versatile effector of hemostasis so that, in physiological conditions, they lead to the clot formation as a response to endothelial lesion. Nevertheless, a platelet disfunction results in an alteration of the process with a consequent thrombotic or hemorrhagic risk. CBC provides the concentration of platelets in blood (normal values 140-450) and the monitoring of this parameter allows the evaluation of mechanical damage on platelets caused by the pump.

Lactate dehydrogenase (LDH) is an enzyme present in low concentration in blood and its increment is related to cellular damage or destruction. It is monitored in LVAD patients to detect hemolysis which can anticipate a possible pump thrombosis[62]. The study conducted by Shah et al.[62] demonstrated the higher sensitivity of LDH than fHb as hemolysis indicator and, therefore, as a marker of pump thrombosis. Values of LDH 2.5 higher than the maximum physiological limit (200 U/L) indicate a consistent hemolytic process, followed by the rise of thrombotic risk. Nevertheless, Trivedi et al.[63] underlined that the only increase of LDH in plasma levels cannot univocally determine a thrombotic activity unless it is supported by further tests more specific to pump thrombosis activity, as fibrinogen measurements.

Fibrinogen (Fg), or coagulation factor I (FI), is a plasma glycoprotein crucial in the coagulative cascade, as mentioned previously. High plasma concentrations of Fg (hyperfibrinogenemia) indicate a possible thrombotic risk[63] and so, since LVAD therapy is associated to hyperfibrinogenemia, the monitoring of this parameter is an integral part of clinical diagnosis for LVAD recipients.

D-Dimer (DD) is a protein fragment deriving from fibrin degradation and it is detectable in blood after the occurrence of fibrinolysis. DD concentration is highly sensitive to thrombus formation, thus high values of DD in LVAD patients can indicate a possible thrombotic

event. Antiplatelet and thrombolytic treatments increase DD levels, while anticoagulant therapy reduce them[61].

Antithrombin (AT-III) is the main physiological inhibitor in the coagulation process, it is synthesized in liver and it can link to thrombin or other coagulation factors (Xa, IXa, XIa) to inhibit the transformation of fibrinogen into fibrin. A lack of this protein can bring easily to thrombus formation. Thereby, it is an important marker for LVAD patients to observe and prevent clot and subsequent thrombus formation.

Prothrombin Time (PT) is a measure of coagulation extrinsic pathway and it determines the coagulative tendency of blood. Consequently, it is useful to adapt the dosage of anticoagulants which act as vitamin K antagonist (i.e. Warfarin) and to evaluate the presence of sufficient quantities of coagulation factors I, II, V, VII, and X. The value of PT related to a patient varies according to the analytic system utilized in a specific laboratory and it depends on the existent variation among tissue factors used in the test reagent and on the non-standardized protocol relative to their measurement.

Partial Thromboplastin Time (PTT) provides information about the intrinsic pathway of coagulation and it is utilized to evaluate the heparin effect on the patient's coagulative system and the levels of factors VIII, IX, XI, and XII in blood.

International Normalized Ratio (INR) is defined as the ratio of patient's PT to a control PT assumed as reference, allowing a standardization of PT value so that it can be compared among different laboratories. A low value of INR indicates a faster coagulative process, hence a thrombotic tendency of the patient, while high values are related to a concrete probability of bleeding. To date, the suggested INR target range is 1.5-2.5 in CF LVAD patients although not all laboratory values below the therapeutic INR range will act as a triggering point of thrombosis activity. As a consequence, low therapeutic INR alone is not a good indicator of thrombotic derived events[63].
Moreover, INR is utilized to evaluate anticoagulant drugs' efficacy (i.e. Warfarin).

PT, PTT, and INR are valid parameters to diagnose a hemorrhagic state rather than a thrombotic one since they do not evaluate platelet activity but only the presence and the functionality of coagulation factors. For this reason, they do not give any information about antiplatelet drugs (i.e. ASA) which do not interact with plasma coagulation factors.

2.2 Platelet activity state

As mentioned previously, platelet aggregation plays a decisive role in post-implant complications, especially in thrombotic events. Hereby, in the last years, the attention of clinical and scientific research turned to the identification of a valid test for monitoring platelet activity. For this purpose, the following sections describe both laboratory techniques and clinical analysis used to evaluate in vitro the state of platelet aggregation.

2.2.1 Laboratory tests

Laboratory tests (Table 2.2) for the evaluation of prothrombotic platelet activity can be classified in:

i. tests based on the induction of platelet aggregation and clot formation (static and dynamics tests)
ii. tests based on the quantification of specific markers for platelet activity without the need of aggregation and clot induction (fluorogenic and chromogenic tests)

These tests can be conducted with different types of carrier fluid: whole blood (previously uncoagulated), platelet rich plasma (PRP), platelet poor plasma (PPP), washed platelets obtained through multiple centrifugation of blood sample, or gel filtered platelets (GFP) – platelets suspension without plasma coagulation factors.

Test/Assay	Carrier Fluid	Sample volume	Classification
Light Transmission Aggregometry (LTA)	PRP	250-300 µL	Static
Thromboelastography (TEG)	Whole blood	300 µL	Dynamic - low shear stress
Rotational Thromboelastography (ROTEM)	Whole blood	300 µL	Dynamic - low shear stress
Platelet Function Analysis (PFA)	Whole blood	800 µL	Dynamic - high shear stress
Thrombing Generation Test (TGT)	PRP, PPP	80 µL	Fluorogenic or chromogenic
Flow Cytometry (FC)	Whole blood, PRP, whashed platelets, GFP	5-10 µL	Immunofluorescent
Platelet Activity State assay (PAS assay)	GFP	10 µL	Chromogenic

Table 2.2 – Laboratory tests for evaluation of prothrombotic platelet function.

Light Transmission Aggregometry (LTA)

Light Transmission Aggregometry (LTA) technique assesses the measurement in vitro of the light transmission increase through the optically dense sample of PRP during platelet aggregation, after the addition of the exogenous platelet agonist[64]. During the assay, the PRP becomes clearer because of the precipitation of platelet aggregates. This determines an increase in light transmission through the plasma sample. The device is constituted by a light source, a thermostat to maintain a constant temperature of 37°C during the experiment, a stirrer that allows shaking together the PRP sample and the agonist, and a spectrophotometer connected to a computer. After shaking, the sample is crossed by a light beam and the more the platelets aggregate, the higher is the intensity of light transmitted. The variation in light intensity is measure by a detector and the signal is sent to the computer. The device records the rate and maximal percentage of light increase from 0% (maximal optical density of PRP, lower bound) to 100% (no optical density of autologous PPP, upper bound) by a photometer. Then the signal is converted automatically in a graphic curve (Figure 2.1) that parallels the increase in light transmission during the platelet aggregation. The slope of the curve, the maximal extent of aggregation (%), and the latency time (lag phase) are the parameters automatically measured and to the PRP sample different agonists are added to stimulate different platelet activation pathways, obtaining information about the several features of platelet function[64,65].

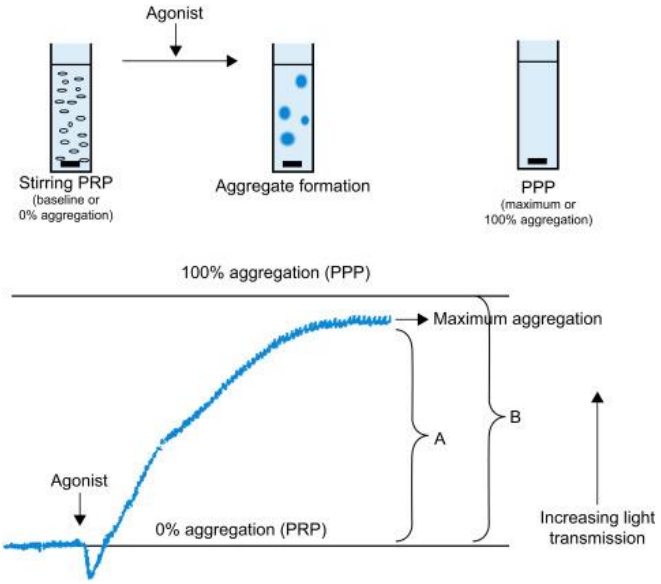


Figure 2.1 – Platelet aggregation curve.

From Figure 2.1 it can be observed an initial and transitional reduction of aggregation, followed by a considerable increment. This shape of the curve depends on platelets behaviour: when the agonist is added, they firstly activate and change their shape, then they start to aggregate increasing the intensity of transmitted light[65].

Although LTA is accepted as a complete assay that clinical laboratories can perform to diagnose platelet function disorders, it presents some peculiar problems. Indeed, the technique may be affected by different preanalytical conditions as the type of anticoagulant, a possible hemolysis of the sample, or low platelet count, and by different procedural conditions as PRP preparation, and the use of different concentrations of agonists. In the case of study, the main limitations regard the fact that platelet response depends only on the interaction with an agonist and not on shear stress. It represents, indeed, the main obstacle for the application of the test in monitoring LVAD patients, since VAD supraphysiological shear stress acting on platelets represents the trigger for platelet activation, which is strictly related the thrombotic risk.

Thromboelastography (TEG)

Thromboelastography assesses viscoelastic changes in clotting whole blood under low shear conditions after adding a specific coagulation activator. It consists on the evaluation of viscoelastic properties of the sample which vary in response to clot formation and/or

dissolution for fibrinolysis[66]. The device (Figure 2.2) is constituted by two measuring channels, allowing to perform simultaneous tests, an electromechanical transducer for motion and a computer for signal processing. The sample is contained in a cup with a suspended metallic pin connected to a torsion wire. After the addiction of activators, the cup is subjected to rotation and the pin stays still until blood remains liquid. When the clot formation starts, fibrin filaments transfer the force from the cup to the pin. The viscoelastic (tensile) force between the cup and the immersed pin results from the interaction between activated platelet glycoprotein IIb/IIIa receptors and polymerizing fibrin during endogenous thrombin generation and fibrin degradation by fibrinolysis[67].

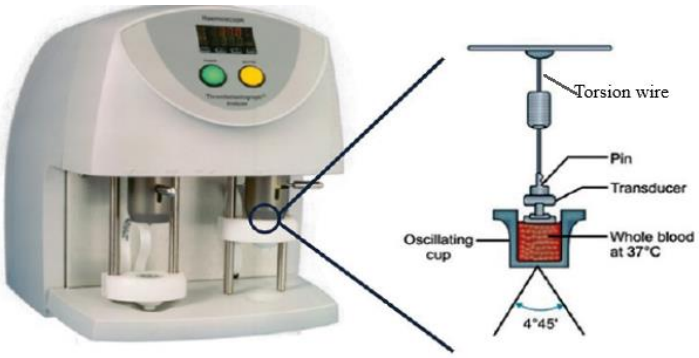


Figure 2.2 – TEG 5000 (Haemonetics, USA).

The movement of the pin is detected by the transducer and transmitted to the computer that returns as output a curve called thromboelastogram (Figure 2.3). It represents clot formation and the eventual fibrinolysis during time, and it provides useful information as clot formation time (K), the maximum force of the clot itself (MA), and the angle α representing clotting velocity.

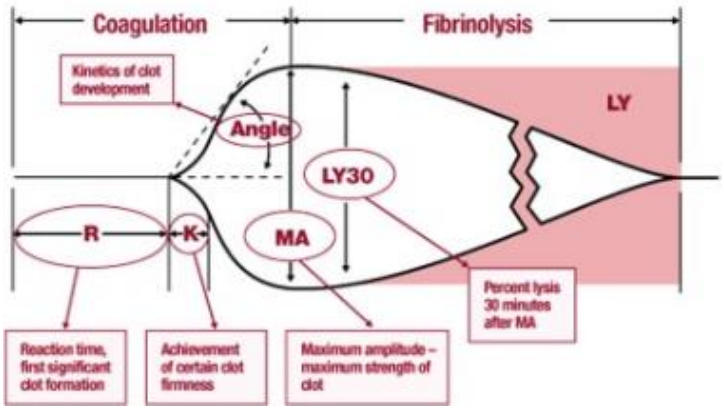


Figure 2.3 – TEG output demonstrating clot initiation, propagation, stabilization, and lysis[67].

TEG is a not complex technique, it can be performed on a whole blood sample, and it can be helpful in the identification of a specific pharmacological treatment after a bleeding event. However, it has some limitations: the main drawbacks are its instability to vibrations and mechanical shock due to the pin suspension and, considering LVAD patients, it is insensitive to oral anticoagulants. These aspects constitute the main reason of the change to the use of rotational thromboelastography (ROTEM)[68].

Rotational Thromboelastography (ROTEM)

ROTEM is a modern modification of TEG technology, in fact it provides a visual assessment of clot formation and subsequent lysis, under low shear conditions (0.1/sec), by measuring and displaying the amount of a continuously applied rotational force that is transmitted to an electromechanical transduction system by developing clot. The operating principle of ROTEM (Figure 2.4) is similar to the one described for TEG with the main difference that here the cup is maintained fixed while a pin suspended on a ball bearing mechanism initially oscillates with an angle of 4° 75′ every 6 seconds through the application of a constant force. As the viscoelastic strength of the clot increases, the rotation of the pin is impeded and is detected optically using a charge coupled device (CDD) image sensor system[67].

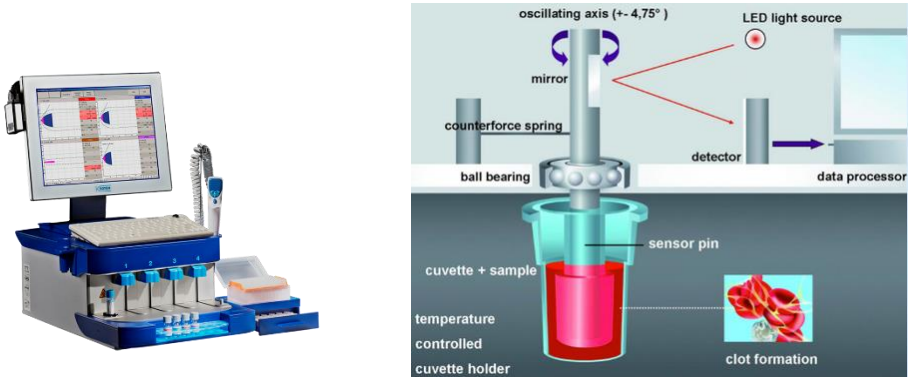


Figure 2.4 – ROTEM Delta (Tem Innovation GmbH, Germany).

The operating characteristics of the device consist in the capability of analyzing 4 samples simultaneously (a standard TEG only 2), an automated pipetting (TEG requires a manual pipetting), and it is not sensitive to vibrations. The output on the computer display is a thromboelastogram (Figure 2.5) which represents clot formation and its eventual lysis in time. Although ROTEM and TEG provide the same information on clot kinetics formation

and strength, their results are not interchangeable since the have different operating characteristics. Clotting time (CT) for ROTEM and reaction rate (R) for TEG are both defined as the time in minutes it takes for the trace to reach an amplitude of 2 mm. Clot formation time (CFT) and kinetics time (K) are defined as the time necessary for clot amplitude to increase from 2 to 20 mm. Angle (α) is determined by creating a tangent line from the point of clot initiation (CT or R) to the slope of the developing curve. Maximum clot firmness (MCF) for ROTEM and maximum amplitude (MA) for TEG are the peak amplitudes (strength) of the clot. For TEG, Lysis 30 and Lysis 60 (LY30 and LY60) are the percent reductions in the area under the TEG curve, assuming MA remains constant, that occur 30 and 60 min after MA is reached. For ROTEM, Lysis Index 30 (LI30) is the percent reduction in MCF that exists when amplitude is measured 30 min after CT is detected[67].

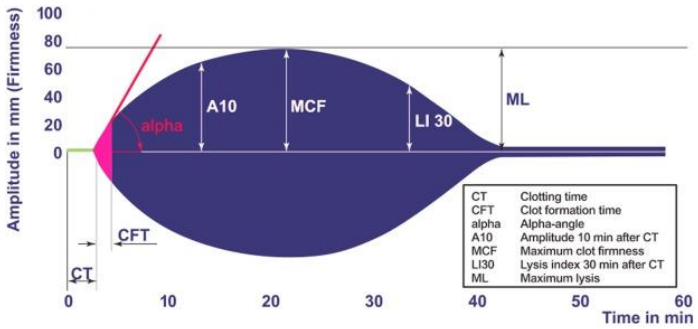


Figure 2.5 – ROTEM output.

ROTEM test offers the same advantages of TEG, with the improvement in the elimination of sensitivity to vibrations and mechanical shocks. Nevertheless, it is unresponsive to anticoagulants and platelet inhibitors[68]. This aspect avoids the evaluation of thrombotic risk in LVAD patients since the instrument is unable to detect residual platelet activity after pharmacological treatment. Indeed, both TEG and ROTEM acquire information on hemostasis but only regarding the hemorrhagic profile: they evaluate the hemorrhagic risk and the associated cause, that regards a functional defect of platelets, which avoids clot formation, or coagulation factors, which induce a premature lysis. However, the thromboelastogram does not provide any information on patient's prothrombotic tendency.

Platelet Function Analysis (PFA)

Platelet Function Analysis measures primary hemostasis in vitro by stimulating in vivo platelet adhesion and aggregation under high shear stress. The most utilized instrument is PFA-100 which is constituted by single use cartridges internally covered by a biochemically active membrane. Whole, citrated venous blood is aspirated through a capillary (Figure 2.6) towards the aperture of a collagen-coated membrane containing either adenosine diphosphate (ADP) or epinephrine (EPI). The presence of these biochemical stimuli, and the high shear rates generated under the standardized flow conditions, result in platelet attachment, activation, and aggregation, slowly building a stable platelet plug at the aperture. The time required to obtain full occlusion of the aperture is called closure time (CT), expressed in seconds, which indicates platelet function in the blood sample[69,70].

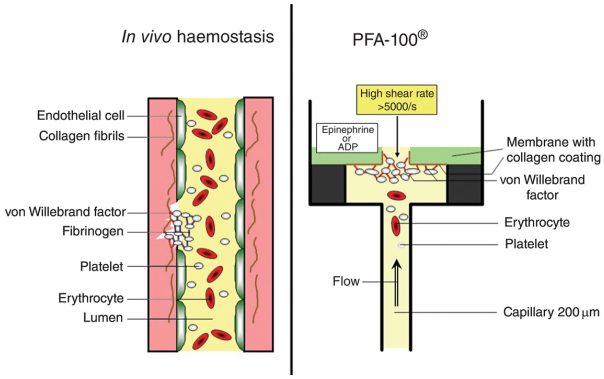


Figure 2.6 – PFA-100 operating principle.

PFA test is a useful tool for evaluating primary hemostasis without the need of a complex sample preparation: the analysis only requires a whole blood sample with no need of further manipulations. Moreover, the volume of blood is reduced, only 800 µL for cartridge. The test is then characterized by simplicity and high sensitivity for hemostatic disorders, such as vWD and platelet dysfunctions (intrinsic or induced by antiplatelet drugs)[70,71,72]. However, this advantage is also the test's main limitation: as the PFA is a global test system for primary hemostasis, it is neither specific to nor predictive of any hemostatic disorder[69]. More specifically, in the case of study, even if the test stimulates platelets subjected to a shear stress with an amplitude comparable to LVAD conditions, it does not replicate the variation dynamics. The analysis is also sensitive to hematocrit and platelet count: hematocrit values

< 35% and platelet count < 15.000 plt/ μL induce a longer closure time; these are considered as threshold values, beyond them the test is not performed. This aspect represents an additional limitation in the setting of LVAD since patients are often characterized by low hematocrit level and platelet count.

The main drawback related to the techniques described above is related to the induction, through a chemical agonist, of clot formation which constitutes the final stage of coagulation cascade. None of them, in fact, focuses the attention only on platelets, which represents the main agent responsible for thrombus formation. Consequently, other tests have been developed with the aim of quantifying the level of platelet activation by the means of the analysis of prothrombotic markers expressed by platelets and related to their activation. They include the following tests described below: flow cytometry, thrombin generation test, and platelet activity state assay.

Flow Cytometry (FC)

Flow cytometry is an analytic method (Figure 2.7) for the quantification and detection of platelet activation markers located on platelet surface through the application of immunofluorescent techniques which provide physical characteristics (dimensions and cellular complexity) and estimate the activation level. In particular through the analysis of platelet surface molecules, as P-selectin, it is able to provide information on the degree of circulating platelet activation and reactivity, diagnose inherited and acquired platelet disorders and monitor antiplatelet agents[73]. Moreover, FC has recently been utilized to analyze both in vitro shear stress induced PA and the level of PA in LVAD patients[74]. Before analysis, platelets are labeled with a fluorescently conjugated monoclonal antibody for the specific antigen, as P-selectin. The cell suspension then passes in the flow chamber through a focused laser beam that activates the platelet which emits three light beams: (i) forward scatter (FSC), related to cell dimensions, (ii) side scatter (SSC), describing cell granularity and complexity, and (iii) fluorescent beam which indicates the presence of a specific antigen on the cell surface previously conjugated. Lastly, the light signal passes through a system of lens and filter to reach a detector so that it is digitalized and sent to a computer.

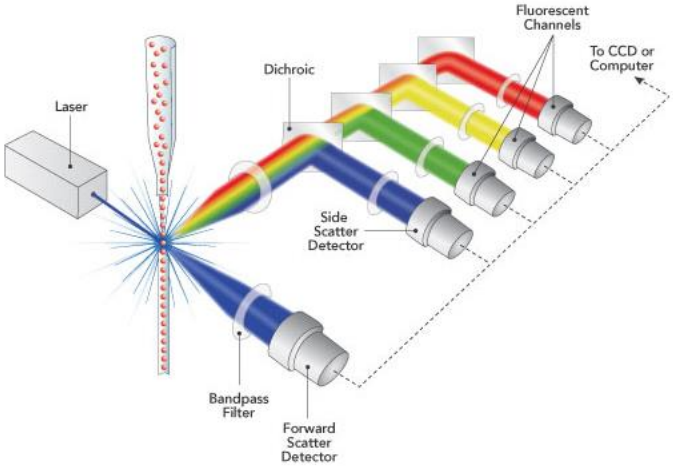


Figure 2.7 – Flow Cytometry components.

Data obtained from FC can be represented graphically through histograms, dot plot, contour plot or density plot on linear or logarithmic scale. Results can be expressed as percentage value, mean fluorescent intensity (MFI) and cellular concentration (cell/µL). The choice of representation depends on the type of information required, qualitative o quantitative.

Although FC is a versatile technology, its complexity requires qualified operators. Moreover, cytofluorimeter is a very expensive instrument which needs a daily calibration to guarantee the accuracy of results and so it is not commonly used in routine clinical practice. An additional drawback regards sample preparation: it needs to be marked by the adding of the conjugated enzyme-coupled antibody, step that has to be done before test execution to avoid PA induced by shear stress due to circulation in the instrument[73,75].

On the other hand, FC has some advantages. Analysis is conducted in a short time and it requires low volumes of the sample (5-10 µL) that can be whole blood, PRP, purified platelets or GFP. The use of whole blood avoids sample manipulation, which could activate platelets, keeping them in a physiological environment. Furthermore, the test is independent from platelet count, since cells individually pass through the interrogation point, and its standardization allows the comparison of results obtained in different laboratories by expressing the analysis in percentage[73].

The wide availability of fluorophores and antibodies allows the execution of multiple FC analysis on different types of cells, representing the gold standard for PA studies. Indeed, FC could be utilized for the evaluation of platelet prothrombotic activity in LVAD patients, but high costs and time required for sample preparation obstacle its application in clinical routine.

Thrombin Generation Test (TGT)

Thrombin Generation Tests (TGT) is a fluorometric assay that enables the quantification of thrombin concentrations in platelet rich plasma (PRP) or platelet poor plasma (PPP), following the addiction of low concentration of tissue factor (TF). The addition of TF, phospholipids (to amplify the effect of TF) and calcium in the plasma, results in coagulation activation and subsequent generation of thrombin. Thrombin then cleaves the fluorescent substrate that is added to the reaction in a later step, releasing a fluorophore whose fluorescence intensity over time is proportional to the concentration of thrombin formed. The separation of a fluorogenic substrate is monitored through the simultaneous comparison with a known thrombin activity in a non-clotting plasma sample[76]. Since plasma sample are used, to exclude contact activation that could increase PA and distort the result, it is necessary to inhibit the intrinsic pathway of coagulation. Therefore, the addiction of TF is essential to reconstruct the coagulation cascade starting from the extrinsic pathway. The output of the assay is a thrombogram (Figure 2.8), a curve characterized by an initiation phase (lag-time) followed by the formation of large thrombin concentrations (propagation), culminating in a peak of thrombin concentration, and terminating with thrombin inhibition by natural anticoagulants[76].

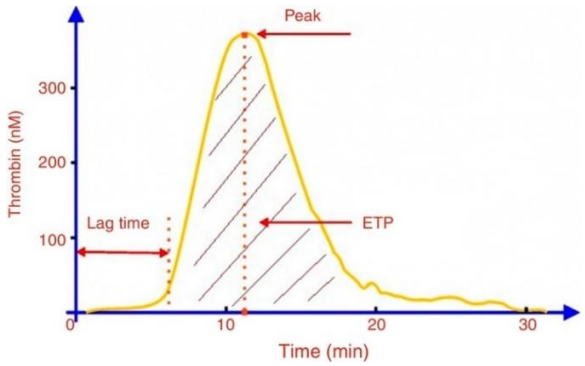


Figure 2.8 – Thrombin calibration curve and its parameters obtained using the CAT method[76].

In figure 2.8, ETP (area under the curve) represents the amount of thrombin generated over 60 min. Clot formation occurs at the end of the lag-time, therefore, the duration of this parameter corresponds to the clotting time. The determination of coagulation state can be derived from analysis of these parameters. A prolonged lag-time and reductions in both ETP and peak values indicate a state of hypocoagulability, characterized by less thrombin

generation. Higher thrombin generation, on the other hand, is characterized by reduced lag-time, and increased ETP and peak values, indicating a hypercoagulable state.
The main limitation of this technique is the non-standardization, in fact the lack of reference values for specific TGT conditions, such as the type and concentration of the triggering agent, or whether or not a contact inhibitor factor is used, makes the use of TGT difficult in clinical laboratories, as it requires appropriate interpretation of the results. Moreover, the output can be strongly altered if the sample derives from a patient treated with anticoagulants. This aspect constitutes a limit in the application of TGT in LVAD patients since they are subjected to antithrombotic therapy which cause a false reduction of the peak in thrombin curve[77].

Platelet Activity State (PAS) assay

Platelet Activity State (PAS) assay is an analytic method developed by Jesty and Bluestein[78] in 1999 able to quantify platelet activation through the analysis of prothrombotic markers, expressed by platelets, which correlate to the level of activation. PAS assay is a chromogenic test for the quantification in real time of thrombin production, a PA marker universally recognised, by the means of Gel Filtered Platelets (GFP) technique which uses purified platelets (without coagulative plasma factors) as carrier fluid.
Thrombin is a chemical agonist which amplifies the level of PA. Therefore, the elimination of the physiological positive feedback (Figure 2.9) of thrombin towards PA is necessary to establish a direct and unique correlation between the levels of mechanical shear stress acting on platelets and their activation state.

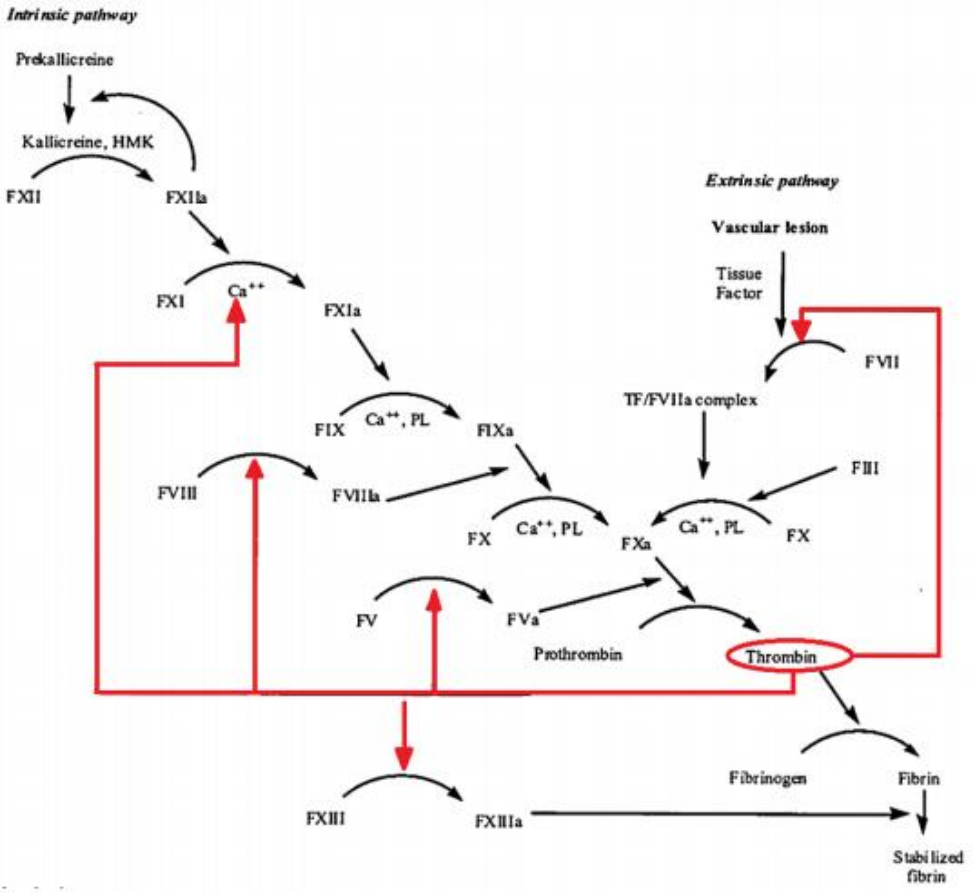


Figure 2.9 – Coagulation cascade and the effects of thrombin positive feedback (red line).

PAS assay utilizes acetylated prothrombin (Ac-FII)[78], a particular prothrombin that can be triggered by prothrombinase complex without altering the level of PA. In particular, GFP samples are exposed to prothrombinase complex factors, as Ca^{++} and FXa, which, in presence of platelets that expose FVa, allow the conversion of Ac-FII in acetylated thrombin (Ac-FIIa)[78]. The action of acetylated prothrombin on coagulation cascade is shown in the following Figure 2.10:

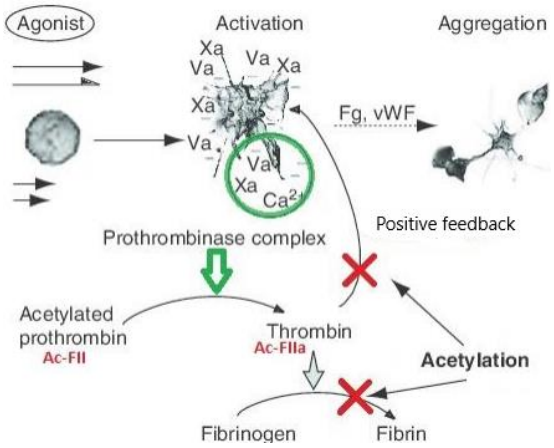


Figure 2.10 – Modified coagulation cascade by Ac-FII.

It can be observed that Ac-FII is activated by prothrombinase complex, as the physiologic FII, but the generation of Ac-FIIa allows both the inhibition of thrombin (FIIa) positive feedback and the conversion of fibrinogen in fibrin[78]. Consequently, the clot formation is blocked, also for the removal of erythrocytes and other coagulation factors from the sample. In fact, the assay is performed on a diluted GFP sample at constant concentration.

The rate of thrombin production can be measured through a chromogenic assay that utilizes chromazine (CH-TH) as specific peptide substrate for thrombin. In detail, a spectrophotometer measures the absorbance level of the sample and the quantity of thrombin produced is determined by the slope of the curve (Figure 2.11) representing the absorbance variation in time: the steeper is the slope, the higher is the production of thrombin that has reacted with CH-TH, and so the level of PA.

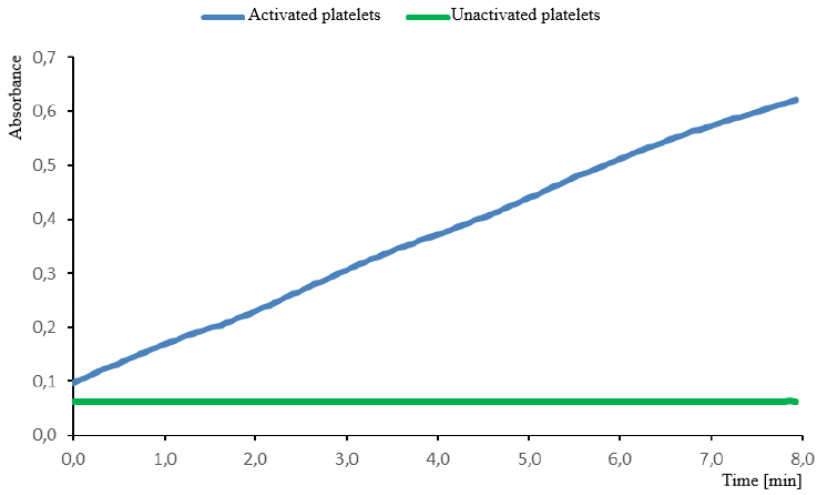


Figure 2.11 – Absorbance-Time curve.

Compared to the tests described above, PAS assay can quantify PA state without the induction of clot formation and the use of chemical agonists which could alter the accuracy of the analysis. Moreover, sample dilution at a constant concentration ensures a test standardization so that data from different laboratories can be compared and, at the same time, they do not depend on platelet concentration in the original sample. The use of GFP as carrier fluid focuses the analysis only on platelets, since it is deprived from all plasmatic components, which, if activated, will express FVa to complete the prothrombinase complex and finally activate FII in FIIa. Hereby, the test correlates the level of PA to the amount of FVa released by platelets. In addition to that, the elimination of the positive feedback using Ac-FII makes the test extremely sensitive to the activation level of the sample. These positive aspects, added to the relative low cost of the test, contributed in the last years to the use of PAS assay for in vitro studies related to the evaluation of platelet activation induced by shear stress in MCS device and they make it suitable for clinical routine.

In the last years, PAS assay has been frequently utilized as an instrument for in vitro evaluation of PA induced by shear stress in LVAD devices, in which platelet response is triggered by non-physiological blood flow produced by the pump[79,80,81,82].

Consolo et al.[79] designed a microfluidic platform able to replicate shear conditions acting on platelets in MSD devices. The collection of GFP samples from the platform allowed the utilization of PAS assay for the quantification of shear-mediated platelet activation over the shear exposure time (Figure 2.12). The analysis demonstrated the sensitivity of PAS assay in the diagnosis of thrombotic risk related to the pump and highlighted the feasibility of the test integration on-chip for the quantification of PA. This feature opens to a new scenario in which microfluidic platforms could be used as a stand-alone point of care devices.

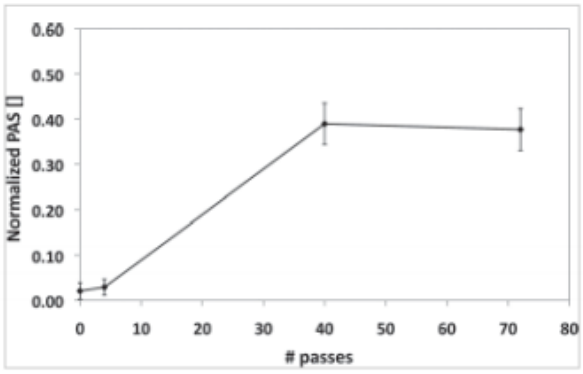


Figure 2.12 – Dynamics of platelet activation (PA) obtained through the PAS assay at different number of passes through microchannels replicating the hyper shear condition[79].

Furthermore, Consolo et al.[81] evaluated in vitro the dynamic of shear-mediated platelet activation induce by hemodynamic shear stress patterns which replicate shear stress profiles of blood recirculating devices (BRDs). The associated levels of PA were quantified with PAS assay, through the analysis of platelet response to repetitive mechanical loading associated with the principal frequency components of shear stress wave forms typical of BRDs. Results showed that high frequency oscillations are the major determinants for triggering the prothrombotic behaviour of platelets whereas low frequency components of the stress signal, with limited oscillations over time, do not induce a significant activation. These findings, provided by PAS assay execution, conducted to a fundamental understanding for the mechanobiological responsiveness of platelet circulating in cardiovascular devices.

In addition, the study conducted by Valerio et al.[82] demonstrated that PAS assay is a sensitive and specific indicator of shear-mediated platelet activation and it is able to spot VAD-related abnormal hematic conditions in clinical setting. They evaluated eight LVAD patients' platelet activation levels using PAS assay and, simultaneously, measuring conventional and prothrombotic hemolysis markers (fibrinogen and lactate dehydrogenase). Reported results (Figure 2.13) shows PA level, detected for 7 of 8 patients, is less than 1% indicating a minimal level of shear-mediated platelet activation. Patient 1, on the contrary, has a significant increase of PAS value following an LVAD associated thrombus formation.

Conversely, LDH and fibrinogen concentrations resulted to be elevated with respect to physiological values (with the exception for fibrinogen level of Patient 6) and without any correlation with PAS values. The study, therefore, demonstrates that LDH and fibrinogen are unable to discriminate between patients suffering of thrombotic events (Patient 1) and other subjects. On the other hand, PAS assay specifically evaluates PA induced by shear stress and, consequently, the thrombotic complications caused by the device.

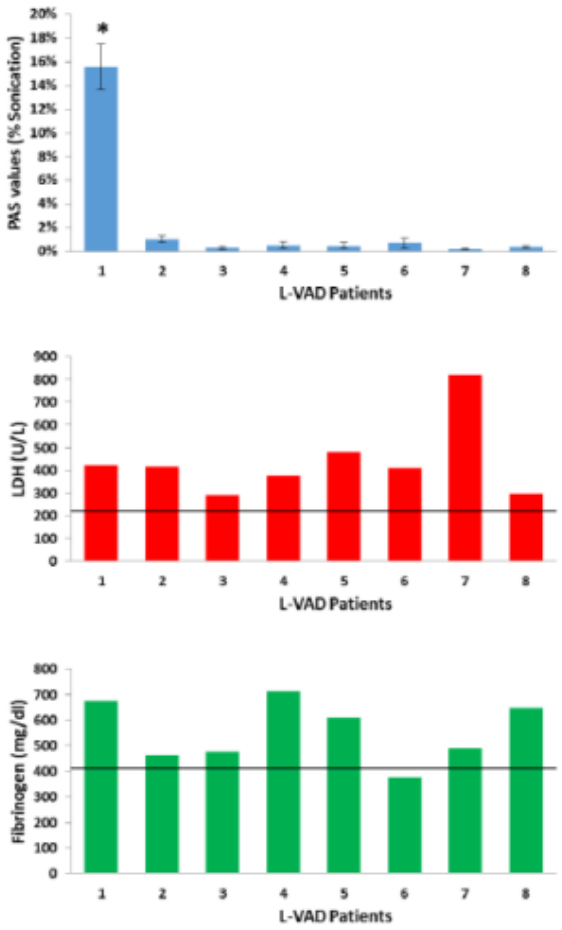


Figure 2.13 – PAS assay, LDH and fibrinogen values obtained for eight LVAD patients.

These studies demonstrate the efficacy of PAS assay in the evaluation of prothrombinase platelet activity, stressing on its potential as a clinically relevant diagnostic test in the setting of LVAD thrombotic complications. These features contributed to the choice of PAS assay for the quantification of PA for the purpose of the present study.

2.2.2 Clinical studies

As mentioned previously, thrombotic complications associated to MCS devices remain a potential catastrophic aspect that contributes significantly to morbidity and mortality of patients. They are principally attributed to non-physiologic blood flow patterns typical to

these devices. In the case of LVADs, the high impeller speeds (axial: 7000-12000, centrifugal: 2000-4000 rpm), applied to achieve physiological cardiac output, generate high turbulent shear-stress levels associated with high dynamic patterns of elevated shear rates. These conditions of supraphysiological shear stress lead to vortex formations, flow separation, adverse pressure gradient, and turbulence which overall contribute to platelet activation[79,81]. This shear-mediated platelet activation (SMPA) shows a dependency from dose and time exposure other than two further effects: (i) platelet damage increases with repetitive passages through the pump, suggesting platelets accumulate cyclic shear stress exposures, (ii) platelets have a sort of "memory", in the sense that even if they are exposed to very high shear stress level for a short time (during passage through the pump), they continue to activate despite subsequent exposure to low shear stress (as encountered downstream of the device)[81].

Based on these considerations, LVAD patients are regularity administered with a combination of antiplatelets and antithrombotic (AT) drugs, to limit the probability of PA initiation mechanisms. However, AT drugs do not completely eliminate the risk of thrombosis and in some cases recipients develop the so-called "drug resistance" or non-responsiveness, meaning that they are insensitive to drug loading and maintenance therapy[83,84]. In addition to that, an incorrect AT administration could lead to under-coagulation, enhancing pump thrombosis, or to over-coagulation, inducing further complications as bleeding vulnerability and acquired Von Willebrand syndrome.

To date, in the clinical setting several blood parameters are considered to evaluate the prothrombotic reaction of platelets in LVAD recipients, their response to AT therapy, and the related risk of thrombosis. Those standard coagulation markers demonstrated, however, partial ability to predict an initial thrombotic condition whose early diagnosis remains a not straightforward procedure; moreover, the monitoring of patients' coagulation status is mainly performed in the pre-operative period, during hospitalization or immediately after while the out-of-hospital home monitoring is still a non-common practice[79,85].

Consequently, clinical studies focus on the use of available laboratory tests to better characterize the effects of antiplatelet therapy on platelet thrombin generation profile.

LTA is the most used method for platelet function and is considered the historical gold standard.

Indeed, Fiore et al.[86] investigated the possible role of LTA in clinical management of CF-LVAD patients. In detail, they utilized LTA to monitor the optimal dosage of Aspirin administered to patients according to the following clinical algorithm applied on PRP drawn from patients: low-dose ASA therapy is administered for 24 to 48 hours after device implant, when basal platelet functions are considered almost normal; then, the aspirin regimen is progressively increased until platelet aggregation, triggered by arachidonic acid, drops below 20%; antiplatelet agent is then adjusted with the use of LTA, according to clinical situation and hemostasis parameters. Results showed that LTA monitoring with adjustment of Aspirin therapy did not increase the rate of thromboses and had no impact on other prognostic factors, as adherence to treatment or procedure-related technical factors.
Moreover, Sugawara et al.[87] analyzed the effect of platelet aggregate formation on the results of LTA. They used citrated blood samples from both healthy volunteers and patients on aspirin or on anticoagulants; platelet aggregate formation was induced by a hematology analysis process, and adenosine diphosphate (ADP) was used as LTA agonist. Then, LTA maximum aggregation percentage (MA%) before and after PA formation was compared, showing a decrease in MA% after PA formation (Figure 2.14).

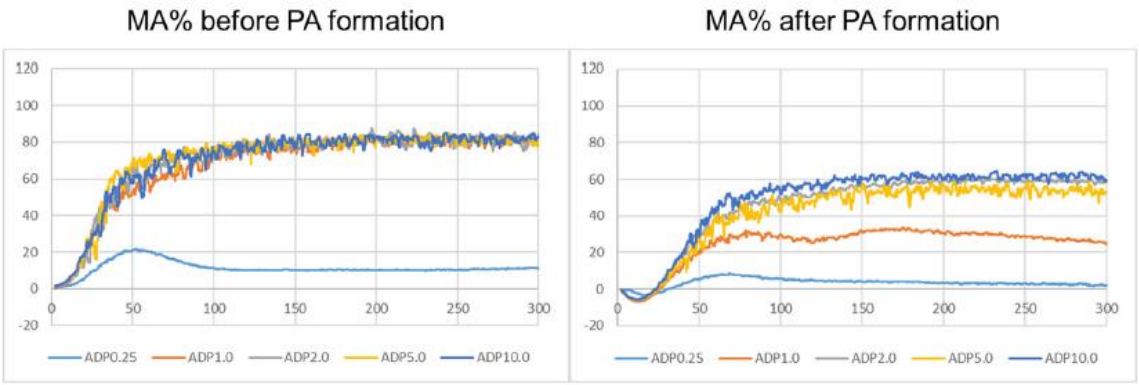


Figure 2.14 – LTA results with ADP before and after PA formation.

Authors attributed these results to the possible PA formation during the centrifugation in the preparation process of the sample which caused the removal of large, activated platelets in PRP, as it is shown in Figure (2.15).

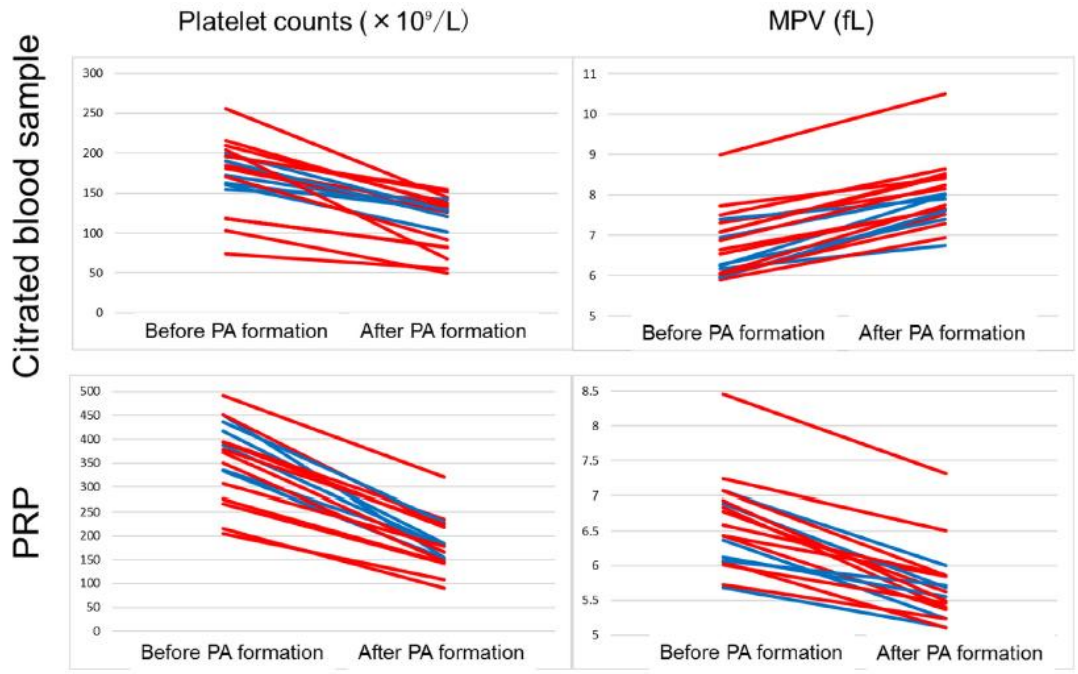


Figure 2.15 – Changes in platelet counts and in mean platelet volume (MVP) after PA. In citrated blood sample platelet counts decreased after the formation of aggregates while MVP increased due to PAs counted as giant platelets. On the contrary, both PRP and MVP decreased after PA formation in PRP samples. MVP reduction is caused by the removal of large, activated platelets during centrifugation.

The study, therefore, determined the interference of platelet aggregate formation in blood samples in the reliability of light transmission aggregometry results. As a consequence, especially in patients with platelet hyperactivity – as could be VAD patients – it has to be confirmed that platelet aggregates have not formed in the sample before LTA analysis.
In the context of the present study, some considerations need to be done. LTA can only form aggregates with the action of an agonist, without the ability to mimic the effects of platelet adhesion, activation, and aggregation on vessel wall damage. This represent a strong limitation since PA is chemically induced, so the test is not able to characterize the activation induced by dynamic shear stress. Moreover, LTA requires a few steps before testing, making it impossible to be a point-of-care test.

Favaloro[70] published an exhaustive seminar on the clinical utility of PFA-100, with a section related on the role of the test in monitoring antiplatelet therapy. PFA is affected by different drugs as well its closure time CT. Moreover, the drug-related effect of CT may depend on PFA-100 test cartridge used. For Aspirin therapy, collagen and epinephrine CEPI CT tends

to be prolonged, but collagen and ADP CADP CT is usually normal. Referring to the study conducted by the International Society on Thrombosis and Haemostasis Scientific Standardization Subcommittee (ISTH SSC)[71], authors are concerned regarding the use of PFA-100 in monitoring Aspirin therapy. They in fact underlined that PFA-100 CT is highly sensitive to vWf binding to platelet membrane glycoproteins under high shear, therefore, a prolonged CT is not always an evidence of aspirin intake but it can also reflect a significant primary hemostatic disorder. Moreover, differences reported in CEPI CT sensitivity for Aspirin depend on the population studied, Aspirin dosage and formulation effect, and variables as the concentration of sodium citrate anticoagulant. They also reported that individuals who appear to be resistant to Aspirin, based on normal CEPI CT on therapy, have COX-1 adequately blocked. This evidence is a consequence of the inverse association between vWf and CEPI CT during ASA therapy, since many patients with arterial disease have high plasma vWf levels which determine a short baseline CT even if they are on Aspirin. In conclusion, both studies agree on the existing evidence that is still premature to use PFA-100 to assess for ASA responsiveness, nonresponsiveness, resistance or treatment failure.

A recent study by Pich et al.[88] evaluated whether TEG maximum amplitude (MA) hypercoagulability (MA $\geq$ 69 mm) at time of LVAD implantation is a predictor of pump thrombosis. TEG-MA hypercoagulability appeared in 10.8% of cases (n = 37 patients with confirmed or suspected pump thrombosis during the study time frame) and in 6.8% of control subjects. However, there was any statistically significance relationship between TEG-MA hypercoagulability and subsequent device thrombosis ($p = 0.46$), suggesting that TEG test is unable to identify LVAD patients at thrombotic risk.

In the last years, several studies utilized PAS assay to evaluate the efficacy of pharmacological therapy in LVAD patients to limit thrombotic and hemorrhagic risk.
Sheriff et al.[89] examined in vitro the antiplatelet effect of Aspirin under the dynamic flow conditions present in VADs with the use of PAS assay. The study highlights the sensitivity of PAS assay to antiplatelet drugs, resulting as an optimal tool for the evaluation of PA in LVAD patients. This allows to the correlation of PA level of a specific patient with the

efficacy of medical therapy administered and/or with the risk of developing thromboembolic complications.

Moreover, Valerio et al.[90] investigated the efficacy of antiplatelet drugs acting as inhibitors on three different pathways: (i) COX (ASA), (ii) phosphodiesterase (dipyridamole, pentoxifylline, cilostazol), (iii) GPIIb-IIIa (eptifibatide). Drug treated GFP was expose to constant or dynamic shear stress profiles and platelet-agent treated response was measured via PAS assay. Results (Figure 2.16) showed that none of the drugs effectively reduces platelet activation after 10 minutes of exposure to shear stress waveforms.

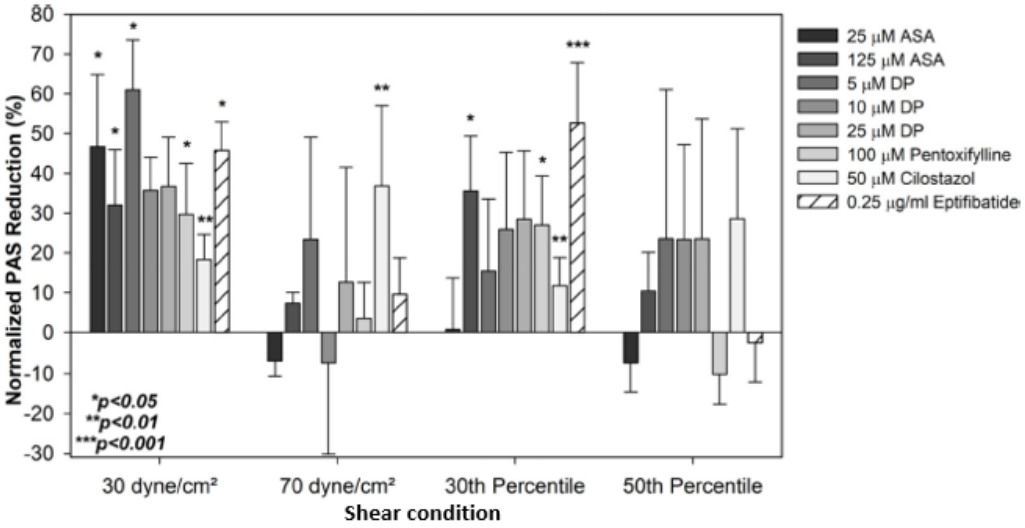


Figure 2.16 – Percentage reduction in drug-treated sheared platelet activation during the 10 minutes of exposure, expressed in terms of ΔPAS, compared to untreated sheared control.

Consolo et al.[91] presented a time-series analysis for the evaluation of shear-mediated platelet activation in LVAD patients who developed thromboembolic events over the time of support. Pas assay was measured: (i) 24 hours after pump thrombosis event; (ii) 30 days after pump exchange; (iii) 60 days after pump exchange; (iv) at 120 days in conjunction with an ischemic stroke; (v) at 240 days when driveline infection occurred; (vi) after heart transplantation (HT). Figure 2.17 reports the dynamics of PAS assay during the time-series analysis.

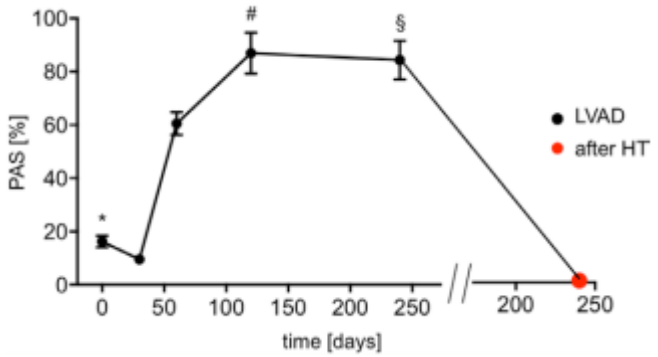


Figure 2.17 – Time-series analysis of PAS assay during LVAD support (black dots) and after heart transplantation (red dot). *Pump thrombosis; #Ischemic stroke; §Driveline infection.

Results demonstrated altered levels of PA during VAD support, suggesting a severe platelet sensitization, due to shear stresses imposed by the pump, which was not reduced or reversed by AT therapy. Conversely, after heart transplantation PAS value rapidly decrease, indicating the restoration of hemodynamic environment after the removing of supraphysiological shear stresses, which allowed the reversal restoration of platelet prothrombotic activity.

These features identify the potential of PAS assay as a reliable biomarker to promote patient-specific pharmacological therapy for the prevention of thromboembolic events in LVAD patients.

2.3 Von Willebrand factor disease

The main risk for bleeding in patients with mechanical circulatory support systems is the acquired Von Willebrand disease (aVWD) related, as mentioned previously, to the high shear stress developed by these devices. In fact, in the case of LVAD, the supraphysiological shear stress imposed by the pump promotes the proteolytic degradation of high molecular weight (HMW) multimers of vWf into smaller fragments of proteins with reduced hemostatic function. Therefore, both the diagnosis of the aVWD and the investigation of molecular degradation mechanisms related to VAD-induced vWf impairment have a crucial role for the prevention of adverse events that may occur in patients.

2.3.1 Laboratory tests

As mentioned in the previous chapter, vWf is a multimeric glycoprotein that plays crucial roles in primary hemostasis as carrier protein for factor VIII (FVIII) to protect it from degradation in plasma, and as linking between clots to injured endothelium through binding sites for platelets and collagen. Considering Figure 2.18, specific binding sites are responsible for its functions: A1 domain changes conformation under shear stress in vivo to allow the binding with platelet glycoprotein Ib (GPIb); A1 and A3 domains are the binding site to collagen, enabling an easier localization of vWf to the site of injury; and D' and D3 domains link the multimer to FVIII[92].

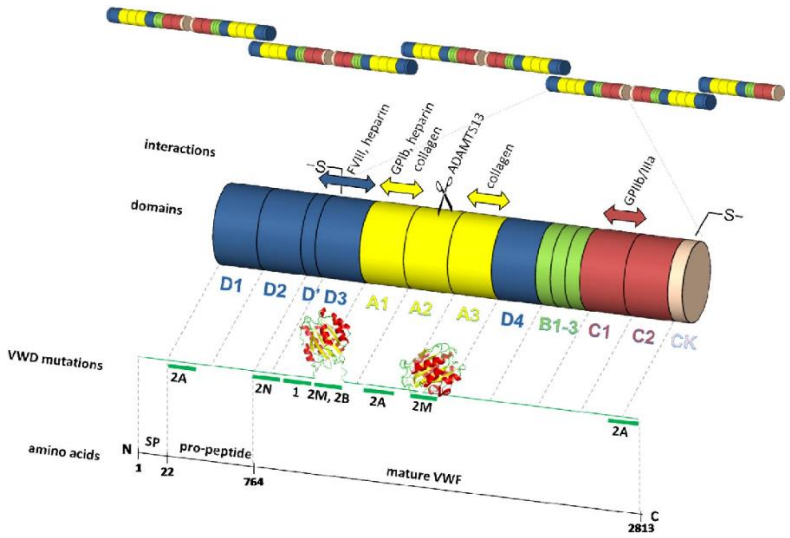


Figure 2.18 – Structure of Von Willebrand factor monomer.

Mutations localized at these sites bring to either a quantitative or qualitative defect in the vWf protein, leading to Von Willebrand disease. The following Table 2.3 summarized the testing panel for the diagnosis of vWD:

Typical diagnostic testing panel	vWf Antigen (vWf:Ag)
	vWf Ristocetin Cofactor (vWf:RCo)
	Factor VIII activity (FVIII:C)
	vWf Multimer distribution
	Ristocetin-induced platelet agglutination (RIPA)
Additional tests	vWf Collagen binding activity (vWf:CBA)
	vWf propeptide (vWfpp)
	vWf gene sequencing
	vWf binding to FVIII (vWf:FVIIIB)

Table 2.3 – vWf laboratory testing.

vWf Antigen (vWf:Ag)

vWf:Ag measures the total amount of the factor present in blood. It is mostly performed by an enzyme-linked immunosorbent assay (ELISA) or by automated latex immunoassay (LIA).

ELISA is a biochemical assay that uses the basic immunology concept of an antigen binding to a specific antibody to allow the detection of small quantities of the antigen itself in a fluid sample. The antigen can be proteins, peptides, hormones, or antibodies.

There are different types of ELISA[93]:

- *Indirect*, the sample that must be analyzed for a specific antigen is immobilized in the fluid phase, generally in 96-well microtiter plates. A primary monoclonal antibody is then added, which is subsequently detected by a secondary, enzyme-coupled antibody. The chromogenic substrate for the enzymes yields a visible color change or fluorescence indicating the presence of the target, whose quantitative or qualitative measure is conducted by colorimetric reading (Figure 2.19). The concentration of primary antibody present in the solution is directly proportional with the intensity of the color.

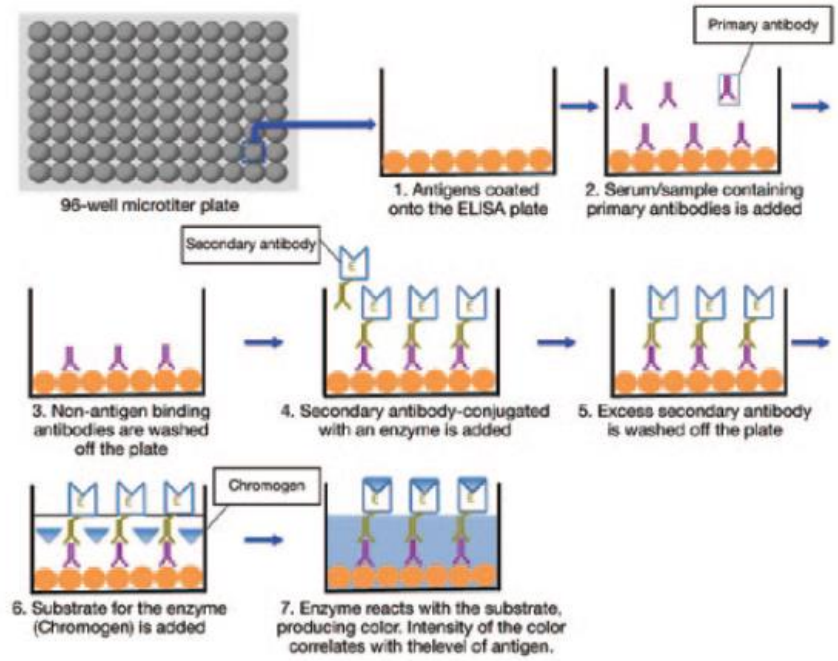


Figure 2.19 – Indirect ELISA technique used to detect a specific antigen in a sample[93].

A main disadvantage of indirect ELISA is the non-specificity of antigen immobilization since different proteins could share the same epitope, so they can bind to the same primary antibody.

- *Sandwich,* this technique is used to identify a specific sample antigen. The well surface is in fact prepared with a known quantity of bound antibody to capture the desired antigen. Then a monoclonal primary antibody is added and it "sandwiches" the antigen. After washing off, enzyme-linked secondary antibodies are applied to bind primary antibody. Unbound antibody-enzyme conjugates are washed off and a substrate is added to enzymatically convert to a color that can be quantified through a spectrophotometer (Figure 2.20).

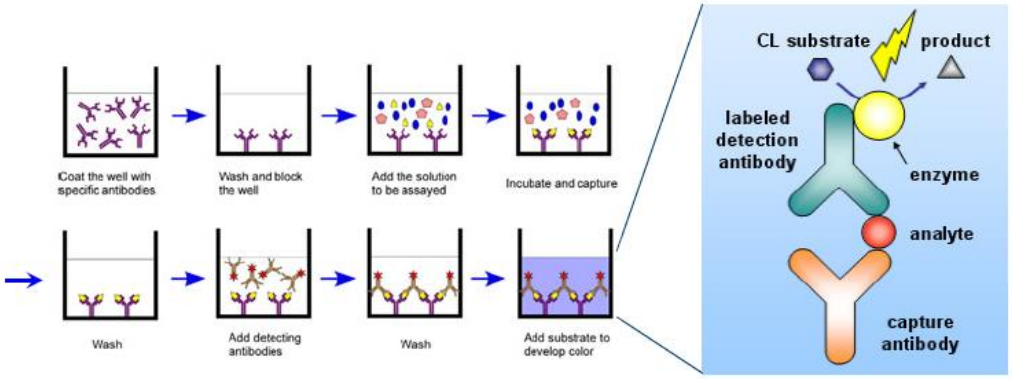


Figure 2.20 – Sandwich ELISA technique.

The main advantage is the use of purified specific antibody to capture antigen since it eliminates the need to purify antigen from a mixture of other antigens, therefore simplifying the assay and increasing its specificity and sensitivity.

- *Competitive*, based on the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. The first step consists in incubating the primary antibody with the antigen in a fluid phase; after that, the resulting antibody-antigen complexes are added to the wells that have been coated with the same antigen: lastly, after the incubation period, any unbound antibody is washed off. Secondary antibody conjugated with an enzyme is added and then a substrate elicits a chromogenic or fluorogenic signal. (Figure 2.21)

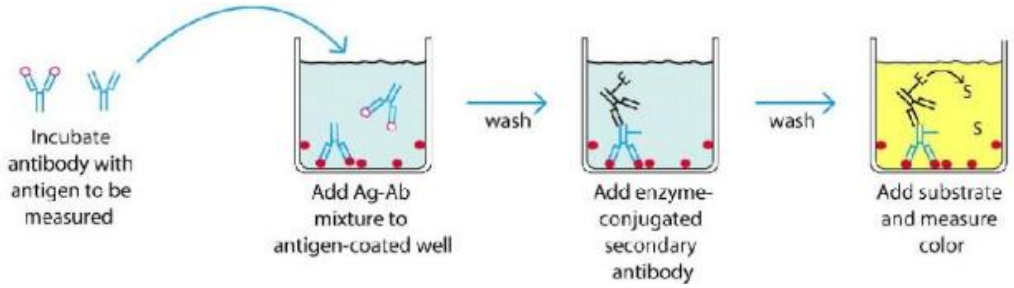


Figure 2.21 – Competitive ELISA.

The main advantage consists of its high sensitivity to compositional differences in complex antigen mixtures, even if when the primary detecting antibody is present in small quantities.

For every type of ELISA, a serial dilution of the purified antigen is loaded in separate wells of the kit to draw a standard or titration curve (contoured in blue in Figure 2.22). To quantify of the antigen in every sample, one locates the color strength of the specific well, in terms of optical density (OD), on the Y-axis that corresponds to the assay measurement of the antigen and follows a line to intersect the standard curve. The corresponding value on X-axis is the concentration of the target antigen (Figure 2.23).

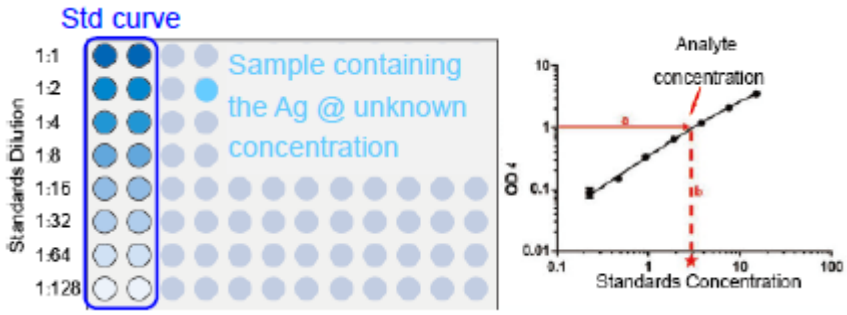


Figure 2.22 – On the left, the wells contoured in blue represent the standard curve; on the right, the black dotted line represents the calibration curve whereas the point on the curve detected by the values on x- and y-axis (red lines) shows the target antigen concentration.

LIA uses latex particles coated with a monoclonal antibody directed against GPIb binding site on the vWf molecule and it is fully automated with a fast turnaround time.
Both of the assays are highly reproducible, fast, with a great analytical accuracy[92].
The major limitations are related to the external factors that influence vWf levels as race, age, pregnancy, and stress. Consequently, vWf:Ag results should be interpreted considering patient history since the normal range is defined as ± a certain percent (usually 2 standard deviations of the average normal value), which means that a non-negligible number of people (2.5% if using 2 standard deviations as cut-off) have in normal conditions a vWf:Ag level below the official lower limit of normal for a given laboratory.

vWf Ristocetin Cofactor Activity (vWf:RCo)

The dosage of ristocetin cofactor activity explores the interaction between vWf and platelet GPIb through the ability of ristocetin antibiotic to agglutinate platelet in formalin at the presence of vWf. In particular, ristocetin introduces a conformational change that enable the factor to bind with platelets through the interaction between vWf A1 domain and platelet GPIb receptor; vWf:RCo is then a surrogate for the ability of Von Willebrand factor to interact with platelets since in vivo this functional activity is triggered by shear stress[92].
Aggregometry methods have however different drawbacks due to their difficult standardization, poor sensitivity to lower levels of vWf, and the non-physiologic ristocetin may introduce another source of variability as sequence variations in the vWf may lead to an altered vWf-RCo binding in the absence of any clinical evidence of vWf malfunction.
Factor VIII activity (FVIII:C)

FVIII is typically tested through the evaluation of its coagulant activity. A one-stage clotting assay is most frequently employed but also two-stage and chromogenic assay can be used. The test results can be expressed as concentration level of FVIII or as a low FVIII/vWf:Ag ratio. Although it is non-specific assay, its concentration is useful for therapeutic monitoring and for having a more complete clinical view[92].

vWf Multimer distribution

Evaluation of vWf multimers with different molecular weights is performed using electrophoresis on an agarose/sodium dodecyl sulfate (SDS) gel run under non reducing conditions. In plasma vWf circulates as a population of molecules with different sizes, ranging from 540 kDa up to 20,000 kDa. Therefore, electrophoretic analysis can distinguish between low, medium, and high molecular weight vWf multimers, which are associated to different bands each one representing a specific molecular weight. The faster moving bands are conventionally indicated as low molecular weight multimers, followed by the intermediate molecular weight multimers, whereas the slower moving bands are indicated as high molecular weight multimers (HMWM). The HMWM are the most effective in mediating platelet adhesion and collagen binding, consequently the absence or decrease of these bands is associated to vWD[94].
The main limitation of this process is related to the complex, labor intensive, and hardly standardizable technique which requires specialized laboratories with resources and expertise available to perform the analysis.

Ristocetin-induced platelet agglutination (RIPA)

RIPA is evaluated using an aggregometer and requires the use of a fresh blood sample, centrifuged right after its collection to obtain a plasma rich in platelets (PRP). The PRP is normalized to 250,000 platelets/μL using the patient platelet poor plasma and then used to assess the threshold value of ristocetin able to induce 30% of platelet agglutination[94]. Normal range is between 0.8 and 1.2 mg/mL of ristocetin. The levels of platelet dependent VWF activity affect the amount of ristocetin necessary to induce agglutination in RIPA, therefore

this relationship must be considered for the evaluation of the patient's condition. RIPA assay is influenced by VWF:Ag levels, the absence of the HMWM, the presence of mutations in the A1 domain and in the GPIb receptor[94].

vWf Collagen binding activity (vWf:CBA)

The vWf:CBA is mainly evaluated through ELISA methods which are sensitive to detect the absence of HMWM and therefore it represents an alternative to the multimer analysis in the initial workup for vWD. Collagen is an important ligand for vWf: types I and III bind to the A3 domain of the factor with and alternative binding site at A1 domain. A lack of larger multimers leads to a reduced activity of vWf to bind the exposed subendothelial collagen, reducing the hemostatic function of the molecule. Consequently, vWf:CBA is considered an additional assay for the evaluation of the factor functionality[92,94] .

vWf propeptide (vWfpp)

The VWFpp (amino acid residues 23-763 of the pre-pro-VWF) is synthesized at the same molar ratio of the mature subunit of VWF (amino acid residues 764-2813). However, most VWFpp molecules circulate in plasma independently from VWF with different half-lives of the VWFpp and VWF (2-3 h and 8-14 h, respectively)[94]. The VWFpp is performed by ELISA with anti-human VWFpp antibodies and its measurement, particularly theVWFpp/VWF:Ag ratio, estimates the rate of clearance of plasma VWF, assuming that the clearance rate of the VWFpp is constant. The evaluation of the VWFpp/VWF:Ag ratio results to be useful to differentiate between congenital and acquired forms of the disease and to monitor, in some acquired vWf syndrome (avWS) patients, the remission of the disease[94].

vWf gene sequencing

The advent of sequencing technologies allowed an easier analysis of the huge vWf molecule, which is characterized by 178 Kb and 52 exons. Even if genetic analysis is not necessary for the diagnosis of vWD, since it is extensively investigated by biochemical assays, it can be a

useful and additional tool to understand the causing-disease mutations and therefore reach a complete and decisive diagnosis.
However, there are still some limitations in vWf sequencing regarding the frequency of non-pathologic sequence variations, which limits the usefulness of genetic diagnosis, and the non-identifiability of the mutation in all patients, in particular in ones with a vWf:Ag lower than a specific threshold[92].

vWf binding to FVIII (vWf:FVIIIB)

vWf:FVIIIB is performed using an ELISA assay to evaluate the capacity of vWf in the test plasma to bind an exogenous source of FVIII. This test is useful to diagnose a specific type of vWD and even to identify the asymptomatic carriers. vWf:FVIIIB is affected only by the presence of mutations in the D′ and D3 domains; nevertheless, in some acquired forms secondary to lymphoproliferative diseases, there is a mildly reduced VWF:FVIIIB, probably due to the presence of anti-VWF antibodies[94].

The heterogeneity of vWD requires several different assays to obtain a complete characterization of the biological condition, as vWf plasma levels and activity, so to establish the cause of the disease and its most adequate treatment. In addition, results must be interpreted considering the context of the individual patient, his personal and family bleeding history, and their consistency needs to be assessed with different assays that sometimes need to be repeated more than once.
Although these limitations and the advent of molecular laboratory techniques, that has become more available in diagnostic laboratories adding a new and more reliable approach to the detection and treatment of vWD, the plasma-based assays will be continuously needed to prove at the biochemical level the sign of mutation(s).

The present study utilizes vWf:CBA via ELISA to assess the activity of Von Willebrand factor. This decision was taken because of the following considerations:

- vWf:Ag test alone, although its high reproducibility, it is influenced by different factors as race, age, pregnancy, stress and physical condition, than could alter the

output. Moreover, it is a test for the evaluation of factor concentration in plasma and not its related activity.

- Aggregometry tests (i.e vWf:RCo) are difficult to reproduce and standardize, and they present a low sensitivity to the lowest level of vWf.
- Multimeric analysis is technically complex, laborious and requires specialized laboratories with appropriate resources and competencies to perform the analysis.

Furthermore, a study conducted by Favaloro[95] demonstrates how vWf:CBA is a useful procedure in the diagnosis of lower factor activity related to its capability in binding collagen. Consequently, since collagen binds more easily HMW multimers, the test is also a valid surrogate of multimer analysis. It can also reduce diagnostic errors, the study in fact shows how the error rate is substantially 2-5 times lower in the collagen binding assay if compared with the standard three tests panel: FVIII, vWf:Ag, and vWf:RCo.

2.3.2 Clinical studies

Literature[57,12,96,97] clearly describes how supraphysiological shear stress imposed by CF-LVAD pumps determine a progressive and irreversible degradation of Von Willebrand factor. This phenomenon – known as acquired Von Willebrand factor syndrome (avWS) – represents a bleeding risk for patients.

Different studies have been conducted to investigate the molecular mechanisms responsible for vWf degradation, in particular Bartoli et al.[12] analyzed whether LVAD-related shear stress and the Von Willebrand factor metalloprotease ADAMTS-13 alter vWf metabolism. They developed an in vitro model to simulate the supraphysiological shear stress imposed by devices that consists in a laboratory vortexer with a velocity of 2400 rpm, shear stress 17.5 Pa, and an exposure time of 4 hours. After the cycling of donors' whole blood in this system, the samples were subjected to electrophoresis and immunoblotting to study vWf degradation profiles and ADAMTS-13 activity. Findings demonstrate there is both a mechanical and an enzymatic degradation of vWf during mechanical circulatory support (Figure 2.23).

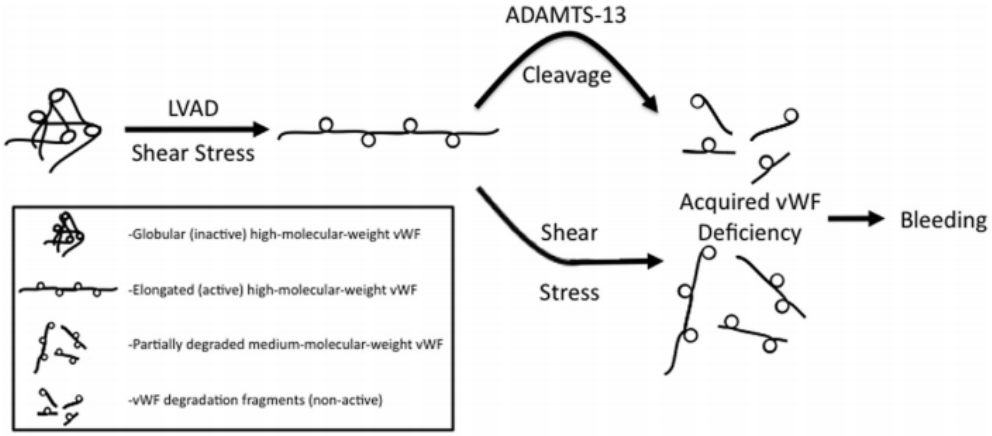


Figure 2.23 - High shear stress during LVAD support triggers globular (inactive) vWF to undergo a conformational elongation into an active form. Then, it alone physically demolishes large, active vWF multimers into smaller vWF multimers. In parallel, ADAMTS-13 cleavage large, active vWF multimers into small, nonactive vWF fragments. As a result, an acquired vWF syndrome predisposes patients with an LVAD to bleeding events[12].

Physiologically, vWF metabolism is regulated by the rheologic conditions of the blood. Specifically, shear stress is an important biomechanical trigger of vWf metabolism: at sites of endothelial damage, turbulence and altered shear stress induce a conformational change of vWf that allows the binding of vWF to subendothelial collagen and platelets. As consequence, there is the formation of a platelet plug at site of injury. During this process, large vWf multimers are most hemostatically active and most responsive to activation by shear stress. However, shear-induced activation also renders vWF more sensitive to proteolytic cleavage by ADAMTS-13, the vWf specific plasma metalloprotease. In the case of LVADs, the abnormal shear stress imposed by the pump is a more powerful trigger to both the mechanical demolition of vWf multimers and the increase activity of ADAMTS-13 that causes a degradation of vWf in small and less active fragments.

Baghai et al.[98] examined avWS in patients with and without LVAD, in the second case patients were analyzed within 14 days and > 30 days after implantation . vWf:Ag, vWg:CBA, vWf:RCo and vWf multimeric analysis were performed. Results (Figure 2.24) showed that avWS is already present in the postoperative state. The decrease in ratios of vWf:CBA/vWf:Ag and vWf:RCo/vWf:Ag in nearly all patients between the time period without LVAD and the early time period indicates a qualitative defect of the factor, traduced in the loss of HMWM, which affects the interaction both with collagen and platelets.

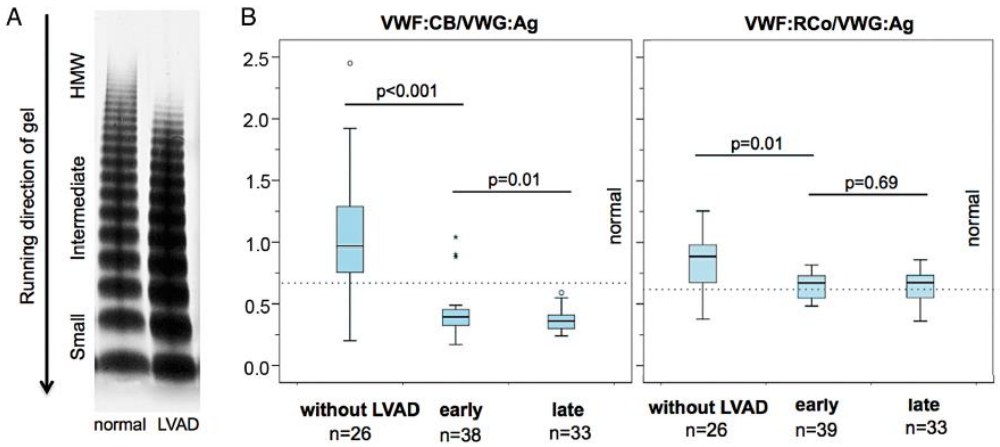


Figure 2.24 – (**A**) Multimeric analysis of vWf performed by gel electrophoresis according to their size on SDS-agarose gel. vWf multimers of an LVAD patient are compared with the sample of a healthy subject. (**B**) Ratios of collagen binding capacity (vWf:CB) and ristocetin cofactor activity (vWf:RCo) to vWf antigen (vWf:Ag).

In this context, Feldmann et al.[99] investigated the perioperative vWF profile through the comparison of an axial pump (HMII) to a centrifugal pump (HVAD) regarding the correlation between perioperative occurrence of avWS, early- and late-postoperative bleeding events. Twenty LVAD patients were tested, 12 had HMII implantation and 8 patients received HVAD whereas 13 patients undergoing coronary artery bypass grafting (CABG) surgery were taken as control group. Blood samples were processed to obtain PPP and vWf constitution was assessed by vWf:Ag assay, and vWf-platelet binding activity level (vWf:Ac); vWf:Ac/vWf:Ag ratio is used to represents the low specific activity of the factor, correlating with the loss of HMWM and therefore is a measure for avWS. Considering Figure 2.25, the study proved that the onset of avWS is either a very early postoperative phenomenon that occurs immediately at the end of surgery or is already present before surgery. Moreover, results show that at the end of surgery the vWF:Ac/vWF:Ag ratio was significantly lower in both CF-LVAD groups compared to the control group, and there are not any significant differences in the postoperative vWF profile while comparing an axial pump (HMII) to a centrifugal pump (HVAD).

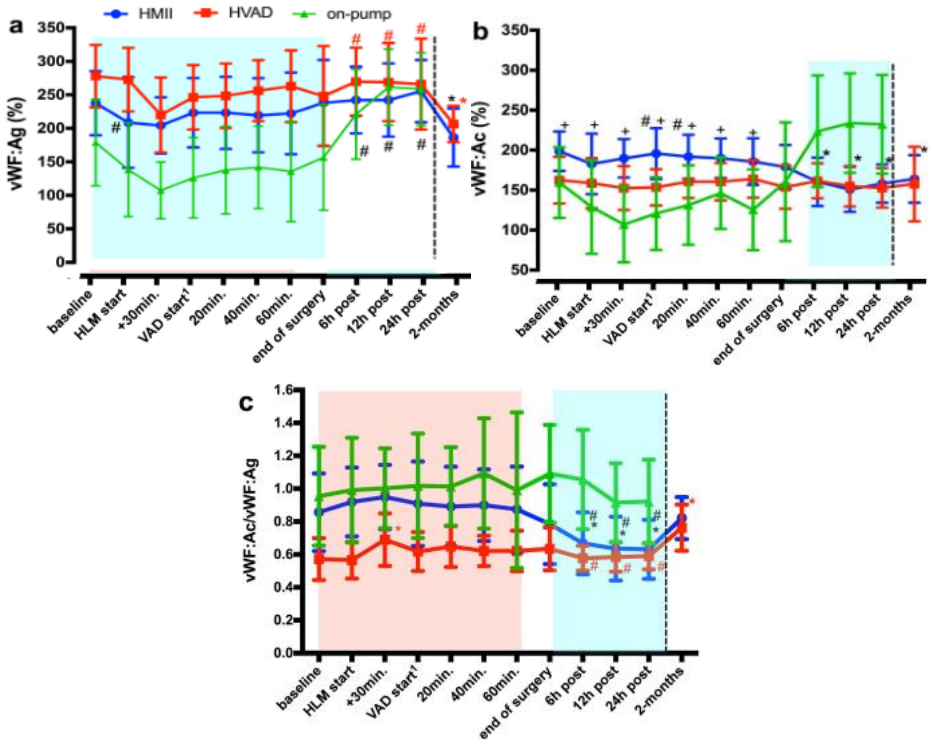


Figure 2.25 – Time course of **(a)** vWf:Ag, **(b)** vWf:Ac, **(c)** vWf:Ac/vWf:Ag ratio in HMII, HVAD, and CABG groups. Symbols: * indicates significance within group compared to baseline value; # indicates significant differences within groups compared to 2-month postop value (Black: HMII group, Red: HVAD group); + indicates significant differences between HMII and CABG; Areas: significant difference as compared to CABG (blue: both CF-LVAD groups; red: HVAD vs. both CABG and HMII).

Furthermore, Bansal et al.[100] conducted a multicentre, comparative, cohort clinical trial to evaluate structural and functional characteristics of vWf in patients with HM3 and HMII pumps. Precisely, 51 patients with HM3 were compared to 29 patients with HMII at 90 days after implantation and the analysis of multimeric content via gel electrophoresis was performed. Results (Figure 2.26) are expressed in terms of vWf HMWM ratio calculated for each band of the gel as the ratio of the fluorescent intensity of HMWM to the low and intermediate multimers.

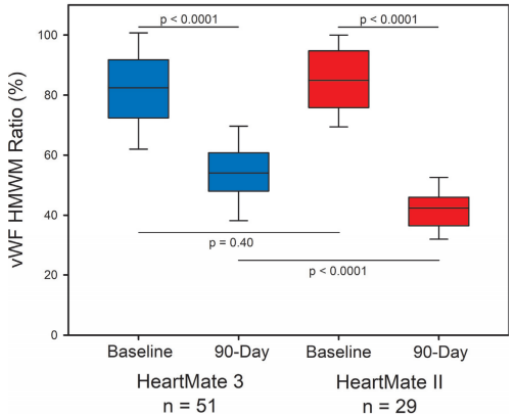


Figure 2.26 - Preservation of vWF HMWMs expressed as a percentage of normalized vWF HMWM ratio after 90 days of implantation and longitudinal assessments with pumps.

As shown, HM3 is associated with a greater preservation of vWF HMWMs than HMII. The different output between the two pumps is related to their mechanical setting: HMII is a second generation axial pump whereas HM3 is a third generation centrifugal pump where the fully magnetically levitating rotor is not in contact with the external structure; this feature eliminates the friction between the components in relative motion, with a consequent reduction of wearing risk and resulting in a more blood-compatible device.

In a recent study, Oezpeker et al.[101] investigated three aspects regarding the relationship between MCS devises and avWS: (i) if avWS is already present in patients with cardiovascular disease before MCS implant; (ii) if MCS implantation influences the development of vWf degradation; (iii) the influence of vWf parameters on clinical outcome. They analyzed plasma samples collected before (≤ 24 h) and after (17.5 days, standard deviation: 5.1 days) MCS implantation, which includes 128 patients with pulsatile MCS implants and 76 patients with CF-LVADs. Von Willebrand factor profile was assessed by analyzing: HMW multimers via electrophoresis, activity via vWf:RCo, and binding capacity via vWf:CBA. The study had the following major findings (Figure 2.27):

- vWf profile was already altered before MCS implantation
- after MCS implantation, vWf profiles further decrease even if in a milder way compared to preoperatively alterations
- changes in several parameters of vWf profile are independently associated with postoperative mortality, indicating they are predictive of a functional decline

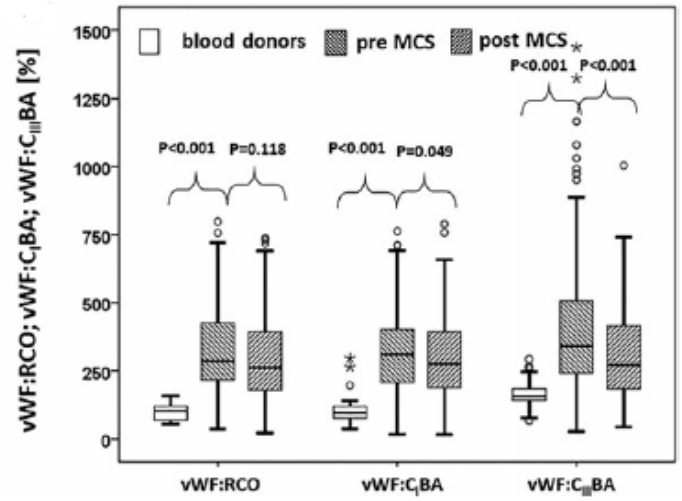


Figure 2.27 – Parameters of vWf activity before and after MCS implantation in comparison to blood donors.

Although the postoperative outcomes related to the bleeding risks and the progress of device technology have improved, the rate of bleeding events remains significant.

Hence, starting from the complex clinical setting of LVAD patients and considering the avWS as one of the major causes of bleeding, it is necessary to consider the antithrombotic therapy administered to patients and its potential contribute to the occurrence of bleeding events.

2.4 Antithrombotic Pharmacological therapy

The annual report from INTERMACS showed that major bleeding is the most common post-operative complications in patients supported by CF-LVADs. In parallel, bleeding is coupled with a prothrombotic risk due to blood contact with titanium surface, supraphysiological shear stress, and activation of systemic inflammatory response.

In the attempt to limit platelet-mediated LVAD thrombosis, patients are subjected to a life-long antithrombotic pharmacologic regimens. Aspirin (acetylsalicylic acid, ASA) is the most used antiplatelet agent and remains the first-choice drug administrated in the long term after VAD implantation. As shown previously, ASA inhibits platelet function by permanently acetylating cyclooxygenase (COX), both COX-1 and COX-2 isoforms, responsible for prostaglandin and thromboxane synthesis. Despite International Society of Heart and Lung

Transplantation provided specific INR range for CF-LVADs, the optimal dose for Aspirin remains wide at 81-325 mg/die according to local practice. In addition, device manufacturers only suggest the use of a specific antiplatelet or anticoagulant agent, without considering the wide range of patients supported by the device.

Indeed, despite technological improvements of the device (greater durability and easier replacement) and the attempt to propose different pharmacological strategies, the occurrence of post-implant thrombosis and bleeding events persists as well the absence of a patient-specific pharmacological therapy.

Different studies have been conducted to provide an overview of different antithrombotic regimens to better understand the relationship between the occurrence of adverse events and the therapy administered, with the aim to find a more patient-specific treatment.

Baumann et al.[13] published a systematic review focused on different antithrombotic strategies administered to patients with CF-LVADs, with both axial and centrifugal pumps. They included 24 studies, reporting an outcome of 2748 LVADs and Aspirin doses varying from none to 325 mg daily. Authors highlighted the unclear role and the lack in standardization of antiplatelet therapy during LVAD support: some studies did not use antiplatelet drugs whereas other studies used dual antiplatelet therapy with Aspirin and dipyridamole or clopidogrel. Overall, 30% of patients suffered long-term bleeding complications during LVAD support, which led to deescalate or suspend antithrombotic therapy. Indeed, if a patient experienced LVAD thrombosis despite an adequate anticoagulation profile, there are limited data relative to the most effective therapy with only some guidance from consensus panel.

At this purpose, Gallo et al.[102] investigated the effect of change in antiplatelet therapy after thrombotic and hemorrhagic events in CF-LVAD patients. The study categorized patients in three groups: (i) high antiplatelet regimen as control group (ASA 325 mg); (ii) low antiplatelet regimen (ASA 81 mg), started after hemorrhagic complications; (iii) double antiplatelet therapy (ASA/clopidogrel), after thrombotic complications. Authors supported the strategy to increase or reduce AT therapy as response to the occurrence of a thrombotic or bleeding event, respectively. Both groups subjected to a variation in medical therapy reported the safety and the freedom from hemorrhagic and thrombotic recurrence. In addition, patients with reduced antiplatelet therapy did not increase thrombotic complication as well patients with increased AT therapy did not increase bleeding complications, if

compared to control group. These results indicate that the reducing or increasing of AT therapy, in response to major hemorrhagic or thrombotic events in LVAD patients, is a safe strategy to avoid recurrences.

Besides these qualitative analysis on the optimal AT therapy for LVAD patients, different studies focused on the efficacy of AT drugs in the mitigation of platelet activation.

Valerio et al.[15] evaluated the effect of ASA on the sheared-mediated platelet response under controlled shear stress conditions. Gel filtered platelets (GFP) were pre-incubated with ASA at two different concentrations (25 μM and 125 μM) and samples for platelet activation measurements were taken at 0, 2, 5, and 10 min after the exposure to shear stress and were quantified via the PAS assay. Stimulation was provided by the hemodynamic shearing device (HSD) and platelets were exposed to constant and dynamic shear stress.

For constant shear conditions, results (Figure 2.28) showed a significant reduction in ΔPAS after 10 minutes of 30 dynes/cm^2 exposure whereas no significant reduction was observed for 70 dynes/cm^2 condition.

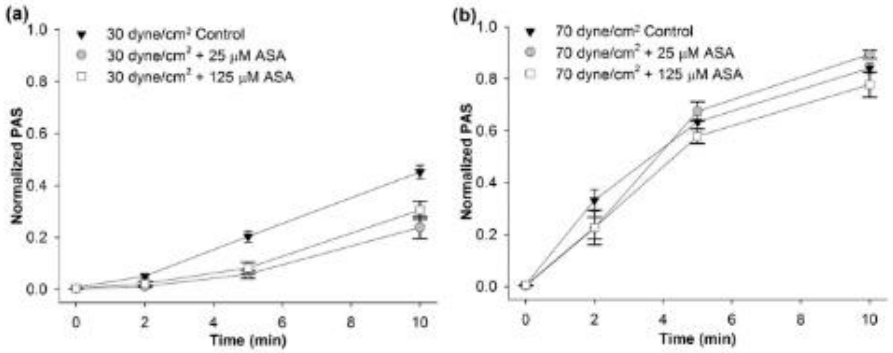


Figure 2.28 – ASA contribution on constant shear-mediated platelet activation. The effect of ASA (25 or 125μM) was analyzed after exposure to constant shear rate values of **(a)** 30 dynes/cm^2 and **(b)** 70 dynes/cm^2.

For the dynamic shear stress treatment, platelets were exposed to waveforms corresponding to 30th and 50th percentile of the probability density function (PDF) of shear stress conditions found in DeBakey VAD. This function describes product of shear stress and exposure time experienced by the platelets and it is calculated along thousands of simulated platelet trajectories. The PDF represents the device thrombogenicity "footprint" and highlights potential thrombotic "hotspot" trajectories. The relative results (Figure 2.29) indicated that only platelets treated with 125 μM of ASA showed a significant reduction in they activity, compared to control, at 30th percentile shear stress waveform exposure.

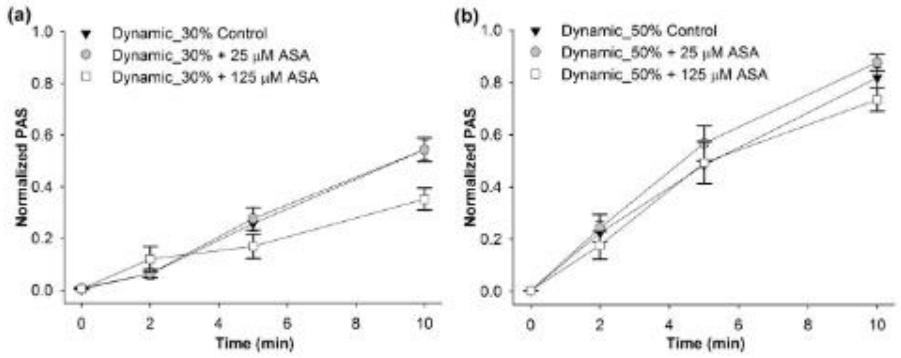


Figure 2.29 - The effect of ASA (25 or 125μM) was investigated after exposure to dynamic shear stress waveforms extracted from CFD analysis of the DeBakey VAD corresponding to the **(a)** 30th (Dynamic_30%) and **(b)** 50th percentile (Dynamic_50%) of the PDF function.

This study clearly demonstrates the limited, or not significant, efficacy of ASA in modulating the shear mediated platelet activation in the case of supraphysiological shear stress exposure and accumulation, which are typical of LVADs. Therefore, these results highlight the necessity of a constant monitoring of ASA administration therapy to control the level of platelet activation and to avoid the occurrence of side effects as bleeding.

Furthermore, Consolo et al.[14] evaluated the effect of different regimens of pharmacological therapy by characterizing the biologic background of platelet-mediated thrombin generation in LVAD patients which were managed with or without ASA. The study provided a mechanistic investigation on the prothrombotic risk and its relationship with platelet function and incidence of adverse events in patients not on ASA. Results revealed a low intensity increase of platelet reactivity in patients not treated with Aspirin, suggesting there are no differences in thrombotic risk between these patients and those managed with Warfarin and Aspirin. Consequently, in LVAD patients, ASA modulation in platelet-mediated thrombin generation mechanisms is low, suggesting that this type of drug is not specific for the prevention of thrombotic risk when PA is induced by supraphysiological shear stress. Moreover, the study highlights a patient-specific tendency to the occurrence of hemostatic disorders.

In the light of these findings, both role and efficacy of antiplatelet therapy have a poor background, and its administration to patients varies widely between institutions and different studies.

Nowadays, the need to balance both arms of coagulation complications (hemorrhagic and thromboembolic) is fundamental to limit the recurrence of adverse events in LVAD patients. This necessity is strongly related to the choice of an antithrombotic therapy that is customized to the patient's current hemostatic and clinical status.

2.5 Aim of the study

Literature widely demonstrates how vWf degradation, platelet activation, and antithrombotic (AT) therapy represent risk factors for the occurrence of HRAEs (bleeding and thrombosis) in LVAD patients.

To date, however, the mutual contribution of these three factors is poorly described.

This study aims at analyzing the competing role of these three factors, also focusing on the effect of ASA discontinuation in patients who suffered a bleeding event. In parallel, platelet prothrombinase activity is evaluated to investigate if ASA discontinuation exposes patients to a major thrombotic risk.

In details, the experimental analysis is structured as follows:

- Characterization of vWf degradation at three time points: pre-implant, early-term follow up and long-term follow up
- Quantification of platelet response at three time points: pre-implant, early-term follow up and long-term follow up
- Effects of AT therapy variation following a bleeding event. It will be evaluated the occurrence of hemorrhagic and thromboembolic events after ASA discontinuation.

Chapter 3

Materials and methods

Vita-Salute San Raffaele University (UniSR) of Milan, within the clinical program of HSR, promotes a study aimed at analyzing Left Ventricular Assist Devices to treat advanced heart failure (aHF). Specifically, the study investigates platelet activity in LVAD patients, with the overall aim to: (i) ameliorate the knowledge of the mechanisms at the basis of post-implant thrombotic and hemorrhagic complications; (ii) correlate these data with clinical characteristics of the patient, implanted device, and antithrombotic therapy administered; (iii) improve life quality and survival of LVAD patients. The current study is part of this project and it focuses on the competitive role of Von Willebrand factor degradation, platelet prothrombotic activity, and antithrombotic therapy in the occurrence of bleeding and thrombotic events in LVAD patients.

This chapter describes the population involving patients subjected to the study and the experimental protocols adopted to quantify platelet activation and vWf degradation; moreover, statistical analysis will be presented to compare clinical data with experimental ones collected during the experimental campaign. Laboratory activities have been performed at Platelet Activity State Laboratory (PAS Lab) of San Raffaele Hospital in Milan.

3.1 Study population and data collection

The study involves CF-LVAD patients subjected to antiplatelet and anticoagulant therapy prescribed after their discharge from the hospital. Blood samples, needed for platelet and vWf activity tests, were taken in respect of their clinical status. All patients were treated at San Raffaele Hospital (HSR) and, before participating to the experimental study, they signed an informed consent. The study was approved by the ethic committee of the Institute.

Generally, CF-LVAD recipients are discharged with a prescription for Warfarin (INR target 2.0-2.5) and ASA. The dose for ASA varies according to the executive management of HSR and with the guidelines related to the specific device. Patients implanted with HVAD receive a dose of ASA of 300 mg/die (Ascriptin) while ones with HM3 a dose of 100 mg/die (CaridoAspirin).

However, in specific circumstances and after medical staff evaluations, patients could be discharged without ASA administration; this situation refers to patients with a high hemorrhagic risk and/or patients who developed post-operative hemorrhagic complications. Moreover, to patients who encounter bleeding events during follow-up, it is prescribed a suspension of ASA administration.

Therefore, according to the object of the thesis, study population have been divided in two groups considering whether they have encountered a bleeding event:

i. Patients who have not been subjected to a bleeding event (control group)
ii. Patients who have been subjected to a bleeding event (study group). As mentioned above, after the event, the administration of ASA was suspended

Patient monitoring, which includes clinical, diagnostic, and laboratory data, was conducted:

i. Pre-implant, to define patient-specific basal value of the analyzed variables
ii. Early follow-up, first 3 months post-implant, to monitor the short-term response of the patient to the therapy and the eventual occurrence of adverse events
iii. Long-term follow-up, period after 6 months post-implant, to monitor the long-term response of the patient
iv. After the occurrence of a bleeding event, in the case of the study group, that has determined a change in the pharmacological therapy. In particular, PAS and vWf activity variables were considered within 3 months and after 6 months from the suspension, so that patient response, the effect of ASA suspension, and the occurrence of further bleeding events could be evaluated

For each patient, hematochemical tests from blood routine exams were collected the same day of PAS test and/or registered from the exam papers carried by patient and taken before the follow-up visit.

Regarding plasma samples for vWf activity, blood exams were collected the day of PAS test in a CBC vial which was subsequently centrifugated to separate plasma and stored at –80°C until the day of the ELISA test.

Hematochemical values, including coagulation parameters, utilized for patients involved in the current study are reported in Table 3.1.

Parameters	Acronym	Unit of measure
Hematocrit	Ht	%
Hemoglobin	Hb	g/dL
Platelets	Plt	10^9/L
International Normalized Ratio	INR	Adimensional
Partial Thromboplastin Time	PTT	Ratio
Fibrinogen	Fg	mg/mL

Table 3.1 – Hematochemical values and coagulation parameters registered during the study.

In addition, demographic variables and parameters related to clinical conditions of patients before implant were registered (Table 3.2).

Pre-implant Data	
Age	Years
Sex	M vs F
Heart Failure Etiology	Ischemic or not ischemic
INTERMACS Profile	1, 2, 3, 4
Creatinine	mg/dL
IABP	Yes vs No
Impella	Yes vs No
ECMO	Yes vs No

Table 3.2 – Pre-implant data. IABP: Intra-Aortic Balloon Pump; ECMO: Extra Corporeal Membrane Oxygenator.

Lastly, for each group, thrombotic and hemorrhagic adverse events were registered and their incidence was compared between the two different groups.

3.2 PAS assay experimental protocol

PAS assay experimental protocol involves a sequence of phases to isolate patient's platelets and quantify their activation level. Quantification of PA level can be measured when isolated platelets are exposed to prothrombinase complex, which is reconstructed in vitro through Ac-FII (as described in paragraph 2.2.1), and subsequently by the use of a spectrophotometric lecture of thrombin production rate mediated by platelets.

PA level is then correlated proportionally to the quantity of thrombin measured by the instrument.

PAS assay experimental phases are the following (Figure 3.1):

1. Blood sample collection
2. Whole blood centrifugation and PRP collection
3. GFP extraction through column-gel filtration of PRP
4. Platelet count to measure platelet concentration in GFP so it can be normalized (GFP*)
5. Incubation of GFP* with Ac-FII and the reagents (FXa and Ca^{2+}) necessary to the prothrombinase complex formation, essential for the quantification of the thrombin produced by activated platelets
6. Sonication of an aliquot of GFP* sample: the analysis of the sonicated quantity allows the normalization of PAS results obtained from patient's platelets
7. Spectrophotometric lecture of thrombin production rate related to basal and sonicated samples, using a thrombin-specific chromogenic substrate (Chromozym-TH, and subsequently indicated as CH-TH). Spectrophotometer detects the variation dynamic related to the absorbance value of the sample in a preset time interval: the slope of the line interpolating the absorbance-time points indicates the quantity of thrombin produced by the test sample, that is the PAS value

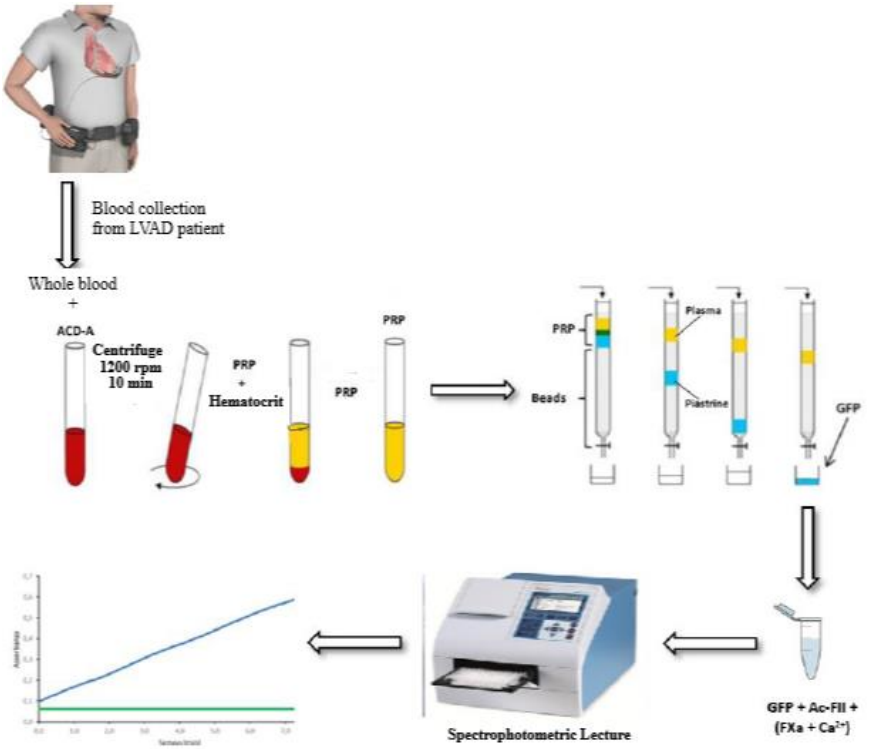


Figure 3.1 – Graphic representation of PAS assay procedure.

Blood sample collection

For each patient, 5 mL of arterial or venous blood are collected ex novo or through a central line. Blood volume is placed inside a Falcon vial containing 500 µL of Anticoagulant Citrate Dextrose Solution (ACD-A) at a ratio of 10:1 (blood volume/ACD-A volume; ACD-A prevents clot formation without altering platelet activation level).

PRP extraction

Blood is centrifuged with a rotation speed of 1200 rpm for 10 minutes without break insertion of the instrument. This procedure allows the separation of erythrocytes and leukocytes from platelet rich plasma (Figure 3.2), ensuring the extraction of PRP.

Figure 3.2 – Example of centrifuged blood sample: separation of platelet rich plasma component (yellow fluid part) from red and white blood cells that precipitate and stratify at the bottom of the vial (red fluid part).

GFP extraction

PRP, extracted from the blood sample previously centrifuged, filters through a column-gel filtration which is preventively filled with a saline solution called *Platelet Buffer* (PB) to guarantee an environment at physiological pH (7.40 ± 0.02). The column contains agarose gel beads (Sepharose 2B, Sigma-Aldrich) whose dimensions retain plasmatic components of PRP – as plasma coagulation factors – favoring the passage of platelets which, moving faster, are the first to elute from the column. Therefore, the filtration process ends with GFP collection, that is platelet suspension deprived from all plasmatic components. GFP transparency is essential to perform the subsequent colorimetric assay which evaluates the absorbance of the sample over time. GFP Separation and collection protocol was previously optimized by PAS Lab researchers.

Platelet count and sample dilution

GFP sample is tested to measure platelet concentration (blood count); the analysis is performed by Sysmex XN-1000 analyzer at hematology laboratory of San Raffaele Hospital. Once the initial concentration is known, the sample is diluted with PB to achieve a standard concentration of 20000 plt/μL. The new sample obtained is called GFP*. The use of a sample with a known and constant concentration of platelets ensures a standardization of the

protocol and a comparison of results derived from different tests and thus different patients. Moreover, the relatively low concentration represents a strength of PAS assay since allows the testing of patients with a low platelet count (common feature of CF-LVAD patients). The preparation for the final sample called EXP, ready to be tested, is completed by the addition to GFP* of $CaCl_2$, a salt that in water environment dissociates in its constituents ions Cl^- and Ca^{2+}; calcium ion in particular is essential for thrombin formation processes.

Sample incubation with Ac-FII

Before obtaining EXP sample (composed by GFP* and $CaCl_2$), a mixture of reagents called *Tube* is prepared and it contains:

- 500 μL of HBS:BSA, a buffer solution containing HEPES 20 mM, NaCl 130 mM, and bovine serum albumin (BSA) at 0.1%
- 100 μL of $CaCl_2$ 50 mM to guarantee the presence of Ca^{2+} ion for coagulation processes
- 100 μL of Ac-FII + HBS:BSA-PEG, in a ratio of 1:4. Ac-FII usage as a precursor of thrombin (FIIa) is, as described in the previous chapter, fundamental to obtain a linear relationship between PA level and thrombin generation. Ac-FII conversion generates indeed a thrombin (Ac-FIIa) unable to activate the positive feedback of coagulation cascade, which would amplify the level of activation and then preventing the correlation (and quantification) of thrombin produced with the real level of PA during the test execution.

Afterwards, 70 μL of this mixture are added to each of four eppendorf (4 for every experiments) which are incubated at 37°C for 10 minutes.

Sonication and PA level normalization

Through sonication, conducted on 350 μL of EXP via a probe sonicator (MIsonix Microson microprobe sonicator Qsonica Llc.) at a power of 20 W for 10 seconds (Figure 3.3), the maximum level of platelet activation relative to a specific patient can be generated (it is considered the 100% of PA). The lecture of the sonicated sample allows the normalization of PAS values.

Figure 3.3 – Sonication of 350 μL of GFP.

Afterwards, 25 μL of the sonicated sample are added in two of the four Eppendorf previously incubated whereas 25 μL taken directly from EXP are added to the two remaining Eppendorf. After this step, 5 μL of FXa are placed in all the four Eppendorf to ensure the prothrombinase complex formation necessary to convert Ac-FII in Ac-FIIa. The resulting samples are incubated at 37°C for 10 minutes.

Spectrophotometric analysis to measure thrombin production

Once the incubation phase of GFP with reagents used to reconstitute prothrombinase complex (Ac-FII, Ca^{2+}, FXa) is concluded, spectrophotometric lecture is carried out in parallel for all the samples prepared. From each one of them, 10 μL are picked up and added in the wells of a microtiter 96-well plate, previously loaded with 150 μL of a solution called *Well* and containing:

- 1 mL of CH-TH (Roche Diagnostics), chromogenic peptidic substrate specific for thrombin
- 2 mL of HBS:BSA-EDTA, which interrupts the thrombin production reaction of the platelet sample, allowing the measurement the rate of thrombin produced during 10 minutes of incubation preceding the lecture. EDTA in fact sequesters Ca^{2+} avoiding the conversion of Ac-FIIa

The dynamic of thrombin production in the sample is measured through a spectrophotometric analysis (Multiskan^{TM} FC Microplate Photometer, ThermoFischer Scientific Inc., Figure 3.4). The lecture protocol is kinetic with a duration time of 7 minutes and the lecture is performed at a wavelength of 405 nm (specific for thrombin bounded to CH-TH). Lecture parameters are set by the user using a software connected to the instrument (SkanIt Software, ThermoFischer Scientific Inc.).

Figure 3.4 - Multiskan^{TM} FC Microplate Photometer, ThermoFischer Scientific Inc.

The output of the lecture is the value of absorbance per minute, which is the slope of the line interpolating the absorbance-time curve acquired: this value represents the quantity of thrombin produced during the incubation time of the sample (10 minutes). Exploiting sonication, PAS value related to the platelet of the patient is normalized with respect to the maximum value of PA, which is specific for each subject.

To guarantee a greater accuracy of the result, for each patient two samples of EXP (EXP1 and EXP2) are produced and they are processed in series; in this way, each experiment has its duplicate. Furthermore, for each EXP, n=8 wells are read (Figure 3.5): considering a line, the first 4 wells contain the basal sample (named controls, C1 and C2, read in duplicate), the following 4 wells contain the stimulated sample vie sonication (named sonicates, S1 and S2, read in duplicate).

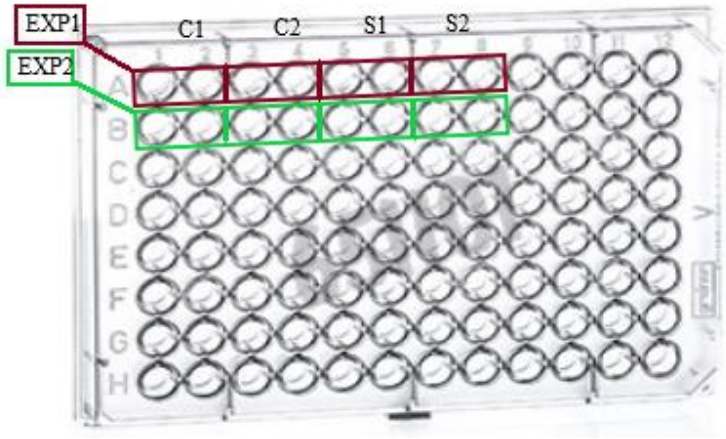
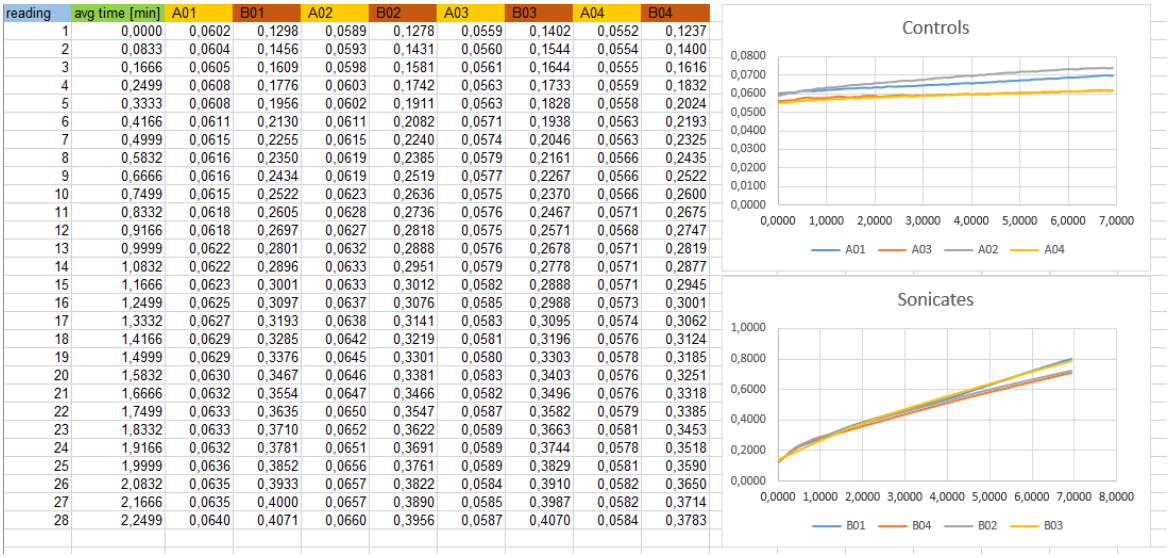


Figure 3.5 – EXP1 wells (in magenta) and EXP2 wells (in green). Both EXP includes controls (first 4 wells named C1 and C2) and sonicates (following 4 wells named S1 and S2).

Before passing to the analysis of the slopes, and so to PAS value, the data obtained are analyzed by the means of spectrophotometric lecture. In particular, it needs to be verified that: (i) the paths of the absorbance-time curve are linear and (ii) they are comparable to each other. In this regard, the software returns an Excel sheet (Figure 3.6) which shows the following features for each experiment:

1. Blue column called reading shows the total number of lectures performed in the established time
2. Green column called avg time [min] represents the lecture rime related to every absorbance value measured
3. Yellow columns indicate absorbance values measured by spectrophotometer and related to the 4 controls (A9, A10, A11, A12)
4. Brown columns indicate absorbance values measured by spectrophotometer and related to the 4 sonicates (B9, B10, B11, B12)
5. The two plots (on the right in Figure 3.6: on the top related to control, on the bottom to sonicates) represents the absorbance-time curves obtained at the end of the lecture

Once verifying that the slopes of the curves are linear and comparable among replicates, both controls and sonicated samples, it is possible to derive PAS value.



reading	avg time [min]	A01	B01	A02	B02	A03	B03	A04	B04
1	0,0000	0,0602	0,1298	0,0589	0,1278	0,0559	0,1402	0,0552	0,1237
2	0,0833	0,0604	0,1456	0,0593	0,1431	0,0560	0,1544	0,0554	0,1400
3	0,1666	0,0605	0,1609	0,0598	0,1581	0,0561	0,1644	0,0555	0,1616
4	0,2499	0,0608	0,1776	0,0603	0,1742	0,0563	0,1733	0,0559	0,1832
5	0,3333	0,0608	0,1956	0,0602	0,1911	0,0563	0,1828	0,0558	0,2024
6	0,4166	0,0611	0,2130	0,0611	0,2082	0,0571	0,1938	0,0563	0,2193
7	0,4999	0,0615	0,2255	0,0615	0,2240	0,0574	0,2046	0,0563	0,2325
8	0,5832	0,0616	0,2350	0,0619	0,2385	0,0579	0,2161	0,0566	0,2435
9	0,6666	0,0616	0,2434	0,0619	0,2519	0,0577	0,2267	0,0566	0,2522
10	0,7499	0,0615	0,2522	0,0623	0,2636	0,0575	0,2370	0,0566	0,2600
11	0,8332	0,0618	0,2605	0,0628	0,2736	0,0576	0,2467	0,0571	0,2675
12	0,9166	0,0618	0,2697	0,0627	0,2818	0,0575	0,2571	0,0568	0,2747
13	0,9999	0,0622	0,2801	0,0632	0,2888	0,0576	0,2678	0,0571	0,2819
14	1,0832	0,0622	0,2896	0,0633	0,2951	0,0579	0,2778	0,0571	0,2877
15	1,1666	0,0623	0,3001	0,0633	0,3012	0,0582	0,2888	0,0571	0,2945
16	1,2499	0,0625	0,3097	0,0637	0,3076	0,0585	0,2988	0,0573	0,3001
17	1,3332	0,0627	0,3193	0,0638	0,3141	0,0583	0,3095	0,0574	0,3062
18	1,4166	0,0629	0,3285	0,0642	0,3219	0,0581	0,3196	0,0576	0,3124
19	1,4999	0,0629	0,3376	0,0645	0,3301	0,0580	0,3303	0,0578	0,3185
20	1,5832	0,0630	0,3467	0,0646	0,3381	0,0583	0,3403	0,0576	0,3251
21	1,6666	0,0632	0,3554	0,0647	0,3466	0,0582	0,3496	0,0576	0,3318
22	1,7499	0,0633	0,3635	0,0650	0,3547	0,0587	0,3582	0,0579	0,3385
23	1,8332	0,0633	0,3710	0,0652	0,3622	0,0589	0,3663	0,0581	0,3453
24	1,9166	0,0632	0,3781	0,0651	0,3691	0,0589	0,3744	0,0578	0,3518
25	1,9999	0,0636	0,3852	0,0656	0,3761	0,0589	0,3829	0,0581	0,3590
26	2,0832	0,0635	0,3933	0,0657	0,3822	0,0584	0,3910	0,0582	0,3650
27	2,1666	0,0635	0,4000	0,0657	0,3890	0,0585	0,3987	0,0582	0,3714
28	2,2499	0,0640	0,4071	0,0660	0,3956	0,0587	0,4070	0,0584	0,3783

Figure 3.6 – Excel spreadsheet providing the verification of absorbance-time curves linearity, obtained via spectrophotometric measurement.

From the Excel spreadsheet obtained as output from spectrophotometer (Figure 3.7), the final value of PAS, expressed in percentage, is calculated as follows:

1. The 8 values of absorbance, related to the 8 wells read for each experiment (4 controls and 4 sonicates), are multiplied for 1000; this step is arbitrary, it is just to improve the readability of output values
2. Mean value calculation of the 8 values of sonicates (Sonic MEAN)
3. Each of the 8 control values is normalized (4 for each EXP) with respect to the Sonic MEAN previously calculated
4. Mean and standard deviation of the 8 PAS values (4 from EXP1 and 4 from EXP2) are calculated. The result, expressed in percentage, represents the PAS value relative to the specific test (highlighted box of Figure 3.7)

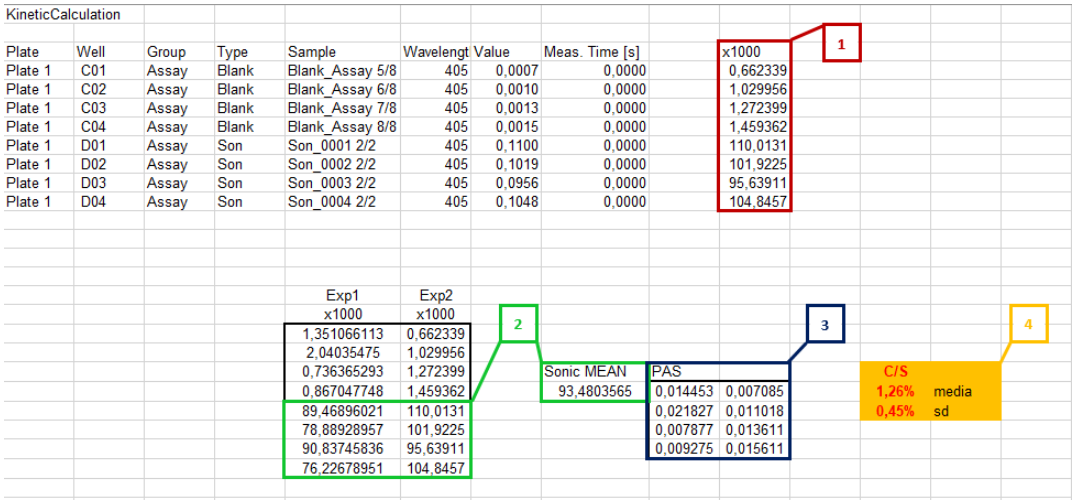


Figure 3.7 – Excel spreadsheet for data processing.

3.3 ELISA vWf:CBA experimental protocol

vWf:CBA assay via ELISA (Asserachrom® vWf:CB, Diagnostica Stago, France) experimental protocol consists in a series of steps to measure the ability of Von Willebrand factor to bind collagen. As mentioned in the previous chapter, this ability is strictly related to HMW multimers of the factor. A reduced rate of vWf:CBA is an indicator of a quantitative or qualitative deficiency of these multimers.

The test principle regards the measurement of vWf, which is captured through HMWM by human collagen coated on the internal walls of a plastic microplate well. Then, rabbit anti-vWf antibodies coupled with peroxidase bind to the remaining free antigens of the bound vWf. The bound enzyme peroxidase is revealed by the addition of tetramethylbenzidine (TMB) substrate. The intensity of the color read by a spectrophotometer is directly proportional to the initial concentration of vWf in the plasma sample.

The protocol is constituted by the following phases:

1. Plasma sample collection
2. Reagents preparation
3. Pre-dilution of samples and calibration curve. Calibration curve is essential to compare the results to standard values, both at the same dilution, so the final concentration of the factor in the plasma can be determined

4. Antigen immobilization. Well strips are covered with human type III collagen from the placenta which can bind a specific domain of vWf so it can be isolated from other plasma components
5. Immobilization of the immunoconjugate. Specific rabbit ant-human vWf antibodies coupled with peroxidase are incubated in the wells so they can bind the protein of interest allowing its detection in the subsequent steps
6. Color development. Tetramethylbenzidine (TMB < 1%) reacts with peroxidase allowing the conversion of the colorless substrate into a colored one, so it can be detected from spectrophotometer
7. Photometric lecture. It calculates the values of absorbance, proportional to the color strength of each well, related to the concentration of vWf

Plasma sample collection

Whole blood from patients is drawn via central line or venipuncture in trisodium citrate anticoagulant tube and centrifuged for 10 minutes at 2500 rpm. Then, plasma is separated, stored at –80°C and thawed the day of the test.

Reagents preparation

ELISA kit, conserved at 4°C, is composed by the following reagents, each one specifically prepared the day of the test:

- Reagent 1 (R1): 16-well strip coated with human type III collagen from placenta are positioned on the plate, numbered, and covered
- Reagent 2 (R2): lyophilized specific rabbit anti-human vWf antibody coupled with peroxidase, it will be reconstituted at step 4
- Reagent 3 (R3): TMB solution, ready to use, is used at step 6
- Reagent 4 (R4): phosphate buffer, it needs to remain at room temperature (RT) 30 minutes before use
- Reagent 5 (R5): 20-fold concentrated washing solution, it needs to be diluted 1:20 with dH_2O. 50 mL of reagent 5 are diluted in 1L of distilled water (dH_2O)

- Reagent 6 (R6): lyophilized human plasma containing, after reconstitution, a known quantity of vWf:CB. It is reconstituted with 500 μL of dH_2O, maintained at RT for 30 minutes, and vortexed before use. It is needed for the construction of calibration curve
- Reagent 7 (R7): lyophilized human plasma containing, after reconstitution, a known quantity of vWf:CB. It is reconstituted with 500 μL of dH_2O, maintained at RT for 30 minutes, and vortexed before use. It is used for quality control of the experiment: once values are obtained, they need to be in the range of assay value insert to ensure all the components of the test work correctly

After that, Eppendorf vials are labeled:

- 5 for calibration curve, each one related to a calibrator value: C_100, C_50, C_25, C_12.5, C_6.25
- 5 for plasma pool curve, each one related to a calibrator value: P_100, P_50, P_25, P_12.5, P_6.25. This curve represents the values associated to healthy patients and so it is an additional standard to compare patients' results.
- 1 for blank assay: B. It represents the background of the experiment and it will be subtracted to the results
- 2 for control quality: CTRL and $CRTL_{1:2}$. The second vial, with a 0.5 dilution coefficient, is the control for the eventual pathological plasmas which may have a lower concentration of vWf.
- N vials, labeled with patient's reference, for the number of patients tested

Then, the scheme of samples disposition is filled in and it will be the same used for photometric lecture.

Pre-dilution of samples and calibration curve

The following procedure is intended for a 1:51 dilution of samples in R4.

- Patients' and pool plasmas are thawed
- 200 μL of R4 are placed in:
    - C_50, C_25, C_12.5, C_6.25
    - B
    - $CTRL_{1:2}$

    - N vials of patients
- Serial dilution of calibration curve, according to the following procedure to obtain the desired calibrator levels:
    - 200 μL of R6 are placed in C_100
    - 200 μL of R6 are placed in C_50
    - 200 μL of C_50 are placed in C_25
    - 200 μL of C_25 are placed in C_12.5
    - 200 μL of C_12.5 are placed in C_6.25
- Serial dilution of control test tubes:
    - 200 μL of R7 in CTRL
    - 200 μL of R7 in $CTRL_{1:2}$
- 200 μL of plasma samples are pipetted into each one of the correspondent N vials, resulting in a pre-dilution factor of 0.5 (200 μL of R4 + 200 μL of plasma)
- 400 μL of R4 are placed in the deepwell, following the scheme previously decided

After that, 8 μL of the content of all Eppendorf vials is pipetted in the deepwell (containing 400 μL of R4).

Antigen immobilization

200 μL of samples are pipetted from the deepwell into the plate composed by precoated wells with type III collagen (Figure 3.9). Then, the plate is covered and positioned on bascule for 1h at RT. This step is necessary for the immobilization of Von Willebrand factor present in plasma samples at the bottom of the well, thanks to the recognition of the factor epitope by collagen coating.

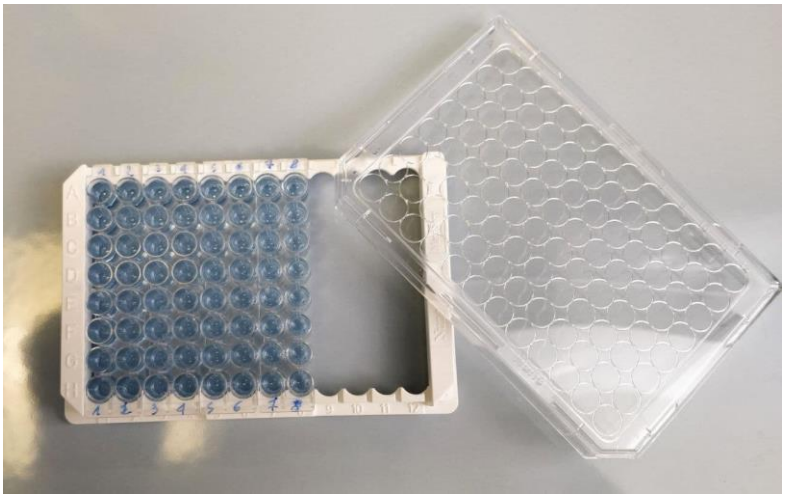


Figure 3.9 – ELISA strips filled with samples for vWf immobilization.

After 40 minutes, R2 is reconstituted with 8 mL of R4 so it can rest for the remaining 20 minutes at RT before being plated.

Immobilization of immunoconjugate

- After 1 hour, plate needs to be washed 5 times:
    - At first, the organic part present in wells is aspired with vacuum pump
    - The plate is then covered with R5 using a 50 mL syringe, gently shaken, and emptied x 5 times. At the end of this passage, only vWf remains in the wells since it is bound to collagen coating, the other plasma components are eliminated
    - If present in wells, bubbles must be eliminated with a tip
- R2 is vortexed and ready to use: 200 µL are dispensed in each well of the plate. R2 will bind to the correspondent epitope on vWf, different from the one specific for collagen; generally, this detection antibody is largely in excess to guarantee the binding with all the antigens present
- The plate is covered and positioned on bascule for 1 h

After 30 minutes, TMB needs to be placed at RT.

Color development

Wells washing procedure is repeated as in the previous step, except the aspiration with the vacuum pump since the organic part was already eliminated.

- 200 μL of TMB are placed in the wells
- The incubation lasts exactly 5 minutes. During this time, TMB reacts with peroxidase enzyme coupled with R2 turning the solution blue colored

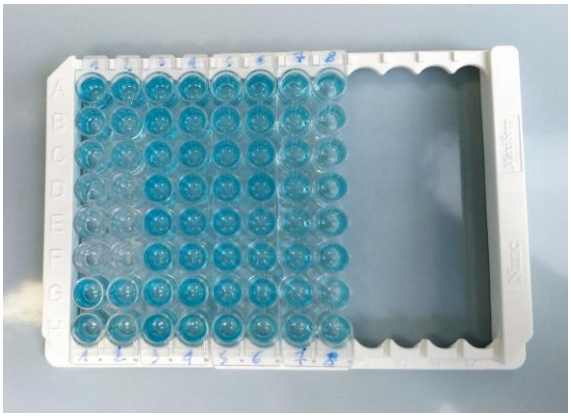


Figure 3.10 – Samples acquire a blue color by the addition of TMB.

- 50 μL of 1 M sulfuric acid (H_2SO_4) is added and the plate is swirled to mix contents. The acid is added to block the reaction and to stabilize the color (Figure 3.11)

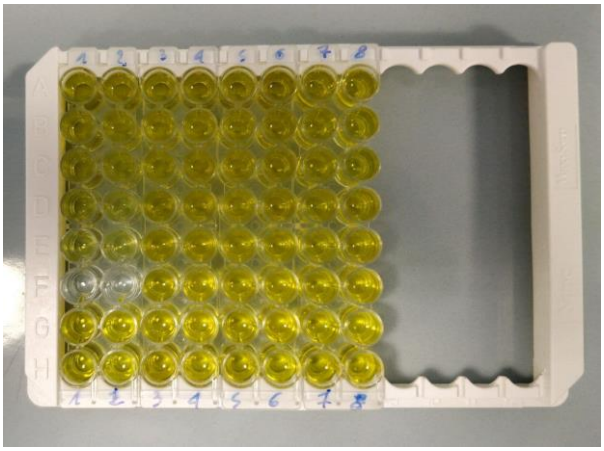


Figure 3.11 – Color changing from blue to yellow after the addition of H_2SO_4 to stop reaction.

- After 15 minutes from the addition of blocking reaction, the absorbance can be measure

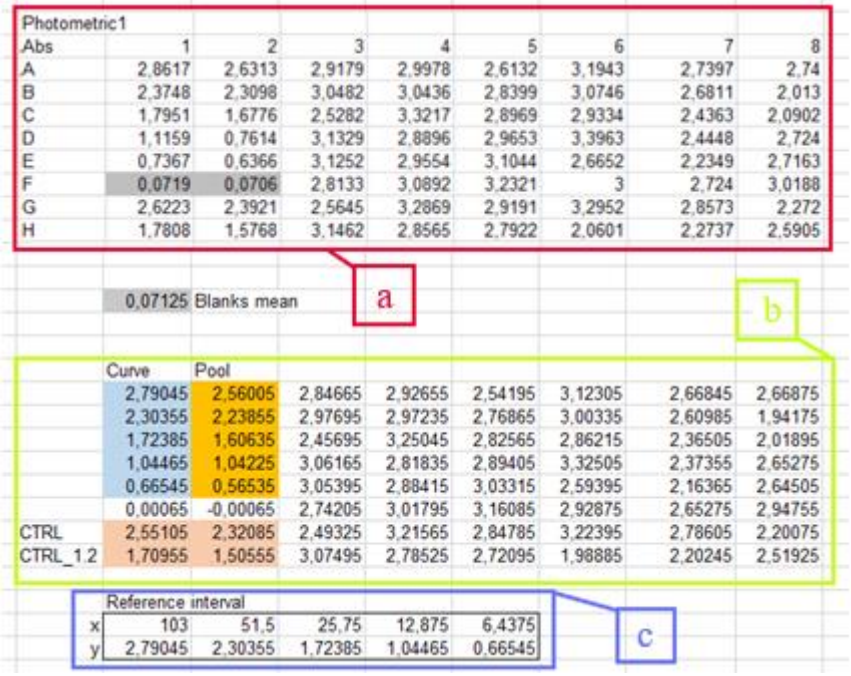
Photometric lecture

Absorbance is measured with a spectrophotometric analysis (Multiskan^{TM} FC Microplate Photometer, ThermoFisher Scientific Inc., Figure 3.4). Lecture protocol is measured at a wavelength of 450 nm and it returns the absorbance of each well which is proportional to its color intensity. Parameters of the lecture are set by user through a software connected to the instrument (SkanIt Software, ThermoFisher Scientific Inc.). Before analyzing the results, representing vWf:CBA of each patient related to the specific absorbance value obtained, data from spectrophotometric analysis need to be elaborated. This procedure can be divided in two steps:

i. Elimination of the background and construction of calibration curve (Figure 3.12)
ii. Data processing and results calculation (Figure 3.13)

<u>Elimination of the background and construction of the calibration curve</u>

The software returns the values of absorbance for each well (Figure 3.12a). Before starting the analysis, the reading must be adjusting to zero by subtracting blanks value that represents the background noise of the plate. At this purpose, blanks mean is calculated between the corresponding wells F1 and F2, and then it is subtracted to the other values, returning a new matrix (Figure 3.12b). The non-highlighted values are the ones obtained from plasma samples of LVAD patients. Afterwards, calibration curve is constructed by considering a reference interval (Figure 3.12c): on the x-axis known calibrator values (%) and on the y-axis the corresponding absorbance values returned by the test are considered.



Photometric1								
Abs	1	2	3	4	5	6	7	8
A	2,8617	2,6313	2,9179	2,9978	2,6132	3,1943	2,7397	2,74
B	2,3748	2,3098	3,0482	3,0436	2,8399	3,0746	2,6811	2,013
C	1,7951	1,6776	2,5282	3,3217	2,8969	2,9334	2,4363	2,0902
D	1,1159	0,7614	3,1329	2,8896	2,9653	3,3963	2,4448	2,724
E	0,7367	0,6366	3,1252	2,9554	3,1044	2,6652	2,2349	2,7163
F	0,0719	0,0706	2,8133	3,0892	3,2321	3	2,724	3,0188
G	2,6223	2,3921	2,5645	3,2869	2,9191	3,2952	2,8573	2,272
H	1,7808	1,5768	3,1462	2,8565	2,7922	2,0601	2,2737	2,5905

0,07125 Blanks mean

a

b

	Curve	Pool						
	2,79045	2,56005	2,84665	2,92655	2,54195	3,12305	2,66845	2,66875
	2,30355	2,23855	2,97695	2,97235	2,76865	3,00335	2,60985	1,94175
	1,72385	1,60635	2,45695	3,25045	2,82565	2,86215	2,36505	2,01895
	1,04465	1,04225	3,06165	2,81835	2,89405	3,32505	2,37355	2,65275
	0,66545	0,56535	3,05395	2,88415	3,03315	2,59395	2,16365	2,64505
	0,00065	-0,00065	2,74205	3,01795	3,16085	2,92875	2,65275	2,94755
CTRL	2,55105	2,32085	2,49325	3,21565	2,84785	3,22395	2,78605	2,20075
CTRL_1.2	1,70955	1,50555	3,07495	2,78525	2,72095	1,98885	2,20245	2,51925

Reference interval

x	103	51,5	25,75	12,875	6,4375
y	2,79045	2,30355	1,72385	1,04465	0,66545

c

Figure 3.12 – Excel spreadsheet for background subtraction and calibration curve values calculation.

Data processing and extrapolation of the vWf activity

Considering the relationship between reference interval and calibrator values obtained, the least squares method is applied. Then, the calibration curve can be plotted in a log-log scale, as suggested by assay protocol (Figure 3.13d): blue point indicators are then interpolated with a logarithmic trend line which resulted to be the best data fitting, as it is shown by its coefficient of determination R^2.

Once the relationship between calibrator concentration and absorbance is determined, it is possible to calculate vWf concentration values of patients. In fact, since both the highest calibrator and the unknown plasmas are tested at the same dilution, results are read directly from calibration curve. This procedure can be seen in Figure 3.13e where for each well, and so for each patient's plasma, x value is determined by substituting the correspondent y value in the calibration curve equation.

The final step consists in correcting the concentration values obtained by the dilution factor: since plasma samples were pre-diluted by a coefficient of 0.5, results were multiplied by 2. The final matrix (Figure 3.13f) contains the results expressed in % related to vWf:CBA of LVAD patients tested.

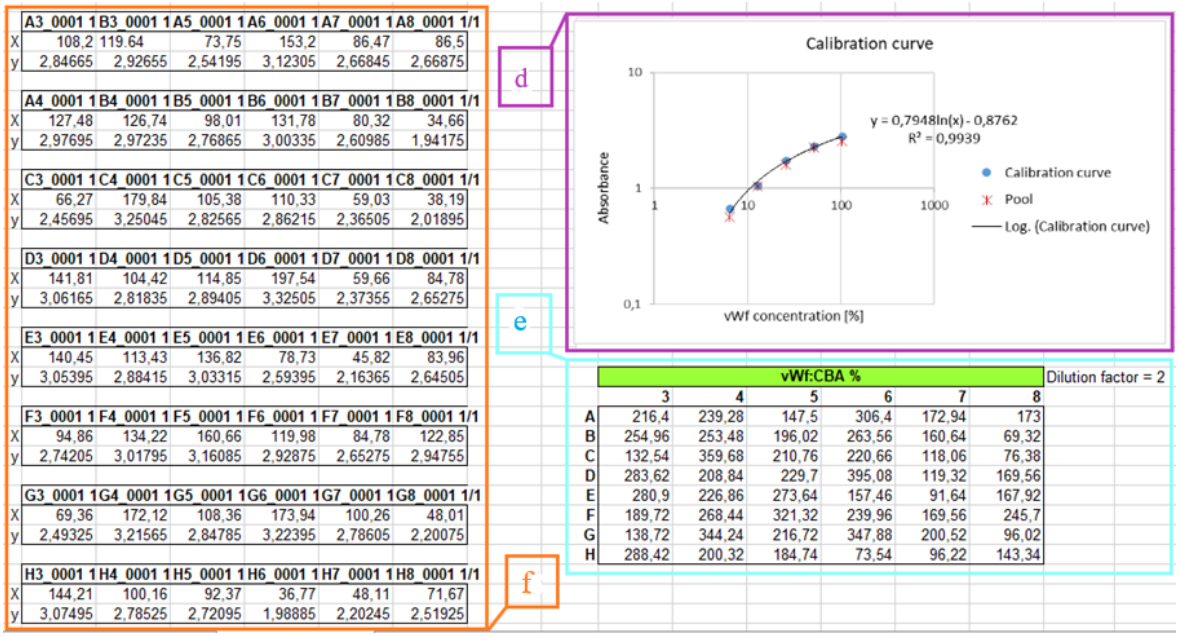


Figure 3.13 – Excel spreadsheet for data processing and results calculation.

3.4 Statistical analysis

Categorical data are presented as absolute numbers and percentages and were compared by the two-tailed χ^2 test or Fisher exact test, when appropriate.

Numerical data are presented as mean $\pm$ standard deviation or median and interquartile range (25^{th}-75^{th} percentiles).

Comparison of variables over the course of LVAD support (i.e. T0, T1, T2) was performed through parametric one-way ANOVA test.

Comparison between groups was performed throughout the parametric T-test of Student or the nonparametric Mann-Whitney U test for normally and non-normally distributed data, respectively.

A p-value < 0.05 was considered statistically significant.

Statistical analysis was performed with the PRISM software v.8 (GraphPad Software, San Diego, CA, USA).

Chapter 4

Results

The current chapter describes the results of the experimental analysis on the combined role of von Willebrand factor degradation, prothrombotic platelet activation, and antithrombotic therapy in the occurrence of HRAEs in LVAD patients. Results will be compared between the two groups at the considered time points, with the aim to evaluate the suspension-related effects of antithrombotic therapy following a bleeding event.

4.1 Patients pre-implant clinical characteristics

Overall, 45 patients were evaluated and the intention to treat was destination therapy for all of them. Data were collected dividing the population in two groups on the basis of the occurrence of a bleeding event: non-bleeding (control group) and bleeding (study group); in detail: control and study groups include 17 (38%) and 28 (62%) patients, respectively. Following the adverse event, 26 (93%) out of 28 patients of the study group suspended ASA administration. Regarding pump model, 26 (58%) patients are on HM3, 17 (38%) on HVAD and 2 (4%) on HMII.

Median time over CF-LVAD support of tested patients is 198.5 days (0-1182 days). Baseline demographic and clinical characteristics of patients are reported in Table 4.1.

Variable	Overall (n = 45)	CONTROL (n = 17, 38%)	STUDY (n = 28, 62%)	p-value
Age at implant (years)	65.1 ± 6.3	60.3 ± 7.7	69.9 ± 4.9	< 0.05
Male sex	41 (91%)	15 (88%)	26 (93%)	> 0.99
Ischemic HF etiology	20 (44%)	8 (47%)	13 (46%)	> 0.99
INTERMACS*				< 0.05
1	6 (13%)	5 (29%)	1 (4%)	
2	8 (18%)	3 (18%)	5 (18%)	
3	24 (53%)	5 (29%)	19 (68%)	
4	6 (13%)	4 (24%)	2 (7 %)	
Temporary MCS*	35 (80%)	15 (88%)	22 (79%)	0.69
Creatinine (mg/dL)	1.4 ± 0.5	1.4 ± 0.6	1.4 ± 0.4	0.81
Hematocrit (%)	35.2 ± 3.7	35.6 ± 3.6	34.8 ± 3.8	0.60
Hemoglobin (g/dL)	11.5 ± 1.1	11.5 ± 1	11.4 ± 1.2	0.80
Platelet count (10^9/L)	182.2 ± 69.5	171.8 ± 48.6	192.5 ± 90.3	0.69
Fibrinogen (mg/dL)	551.8 ± 122.6	591.6 ± 109.9	511.9 ± 135.2	0.12
D-Dimer (μg/mL)	3.6 ± 4.6	2.3 ± 3.5	4.9 ± 5.6	0.06

* Missing data from 1 patient

Table 4.1 – Baseline demographic and preoperative clinical characteristics of tested patients.

Data from Table 4.1 indicate that 41 (91 %) patients are male. The etiology of heart failure was ischemic for 44% of the patients. Moreover, before CF-LVAD support, 35 patients (80%) where stabilized with temporary mechanical circulatory support (Intra-Aortic Balloon Pump, Impella, ExtraCorporeal Membrane Oxygenator). Regarding INTERMACS profiles, 6 (13%) patients are classified in level 1, 8 (18%) in level 2, both describing a greater severity of HF, 24 (53%) in level 3 and 6 (13%) in level 4. There is a statistically significant variation of INTERMACS profiles (p-value < 0.05), indicating a variability in the severity of HF.
The two population groups (control vs. study) are clinically comparable, meaning that there are no statistical differences between the groups with respect to the analyzed variables. The only exception regards the age at implant (p-value < 0.05); mean value related to the age of study group is higher if compared to the one of control group. These values are in agreement

with the division of the two groups: study group involves older patients which are clinically more fragile, resulting to be at a greater risk of experiencing adverse events due to specific comorbidities related to the advanced age.

For each of the two groups, the occurrence of thrombotic and bleeding events was recorded. Some patients developed more than one adverse event over time and, consequently, the number of events could exceed the number of patients affected.

Control group is characterized by patients who did not suffered bleeding events and are subjected to ASA treatment; in this group, 2 (12%) patients developed a thrombotic event.

Thrombotic events registered

- 1 pump thrombosis
- 2 cardioembolic strokes

Study group is characterized by patients who were on ASA and suffered hemorrhagic events. The administration of the drug was suspended for 26 (93%) out 28 patients. Only 1 patient (4 %) developed a thrombotic event before ASA discontinuation.

Bleeding events registered before ASA-discontinuation

- 7 gastrointestinal bleeding
- 1 hematuria
- 1 rectal bleeding
- 2 hemothorax
- 3 anemia
- 10 epistaxis
- 1 bleeding on probing
- 1 low Hb
- 1 intracerebral hemorrhage
- 1 pericardial tamponade

Thrombotic events registered before ASA-discontinuation

- 1 thrombosis inflow cannula

Median time between LVAD implantation and the development of a bleeding event is 126.5 days (15-893 days).

After ASA suspension, further bleeding events were registered for only 5 (19%) out of 26 patients; some patients developed more than one bleeding event over time, resulting in a total of 10 hemorrhagic events. Only 1 patient suffered a thrombotic event.

Bleeding events registered post ASA-discontinuation:

- 3 epistaxis
- 1 gastrointestinal bleeding
- 1 pericardial tamponade
- 1 bleeding on probing
- 3 intracerebral hemorrhage
- 2 anemia

Thrombotic events registered post-ASA discontinuation:

- 1 thrombosis inflow cannula

The comparison between the two group (control vs. study) suggests that:

- ASA discontinuation considerably reduces the recurrence of bleeding events (only 5 (19%) out of 26 patients who suspended ASA suffered further hemorrhages)
- ASA discontinuation does not increase the thrombotic risk (only 1 (4%) patient not on ASA developed a thrombosis).

4.2 Patients post-implant clinical characteristics

Blood and coagulation parameters of patients, corresponding to the early- and long- term follow up, are reported and compared between the two groups in Table 4.2.

Variable	CONTROL (n = 19, 42%)	STUDY (n = 26, 58%)	p-value
Ht (%)			
T1	35.5 ±3.7	36.0 ± 3.9	0.69
T2	38.5 ± 5.5	36.9 ± 3.9	0.40
Hb (g/dL)			
T1	11.5 ± 1.5	11.7 ± 1.5	0.80
T2	12.65 ± 1.9	11.9 ± 1.8	0.22
Platelet count (10^9/L)			
T1	224.8 ± 27.6	242.0 ± 66.2	0.42
T2	205.4 ± 67.8	227.1 ± 78.8	0.38
INR (ratio)			
T1	2.4 ± 0.5	2.2 ± 0.5	0.24
T2	2.4 ± 0.7	2.4 ± 0.7	0.74
aPTT (s)			
T1	35.7 ± 3.9	35.7 ± 8.8	0.97
T2	35.2 ± 2.9	35.5 ± 4.2	0.82
Fb (mg/dL)			
T1	440.9 ± 62.9	433.1 ± 122.8	0.84
T2	409.8 ± 120.7	392.1 ± 102.7	0.62
DD (μg/mL)			
T1	3.1 ± 1.5	3.6 ± 2.4	0.48
T2	1.5 ± 0.5	2.3 ± 1.5	0.09

Table 4.2 – Hematochemical parameters at T1 and T2: CONTROL vs. STUDY.

Results indicate there are not significant differences in the coagulation profile of the two groups. Accordingly, these parameters do not allow to stratify patients at risk of developing HRAEs.

Moreover, coagulation parameters have been compared pre- and post-ASA discontinuation (Table 4.3). Results show no significant variations between the two conditions in terms of AT therapy suspension.

Variable	PRE ASA SUSPENSION	POST ASA SUSPENSION	p-value
Hematocrit (%)	35.7 ± 4.9	36.7 ± 5.5	0.5
Hemoglobin (g/dL)	11.7 ± 1.7	11.8 ± 1.7	0.7
Platelet count (10^9/L)	235.8 ± 76	224.6 ± 73.2	0.5
INR	2.3 ± 0.5	2.3 ± 0.8	0.6
aPTT (s)	35.2 ± 6.4	35.7 ± 6	0.5
Fibrinogen (mg/dL)	416.5 ± 124	396.7 ± 99.2	0.6
D-Dimer (μg/mL)	3.1 ± 2	2.6 ± 2	0.2

Table 4.3 – Coagulation parameters pre- and post-ASA suspension.

4.3 Analysis of von Willebrand factor degradation

The experimental analysis focused on the degradation profile of vWf in the two groups in terms of vWf collagen binding activity via ELISA.

In detail, vWf:CBA assay was performed for both control and study groups at the following time points:

- T0 (pre-implant), to determine the baseline status of the factor and compare it between patients
- T1 (early-term follow up), to investigate how LVAD implantation affected the degradation profile of the factor in the short time
- T2 (long-term follow up), to analyze the impact of LVAD support over time

The study group was also tested at two additional time points to examine the effects of ASA suspension on vWf profile:

- T1* (early-term follow up post ASA suspension)
- T2* (long-term follow up post ASA suspension)

Experimental values on vWf collagen binding activity measured over time via ELISA are reported in Figure 4.1.

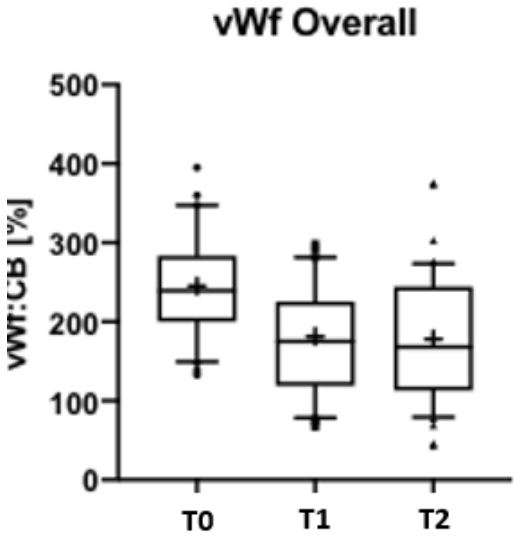


Figure 4.1 – Box plot of vWf collagen binding activity over time; T0: pre-implant, T1: early-term follow up (<3 months); T2: long-term follow up (>12 months) for the overall population. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots, squares, and triangles indicate outliers.

According to the present analysis, vWf:CB activity changed significantly from T0 to T1 (p-value 0.004). Interestingly, vWf:CB at T1 and T2 are comparable (p-value 0.98). Both median values at T1 and T2 were 1.4 times lower than at T0.

Afterwards, vWf:CB activity was compared (Figure 4.2) between the two groups (control vs. study).

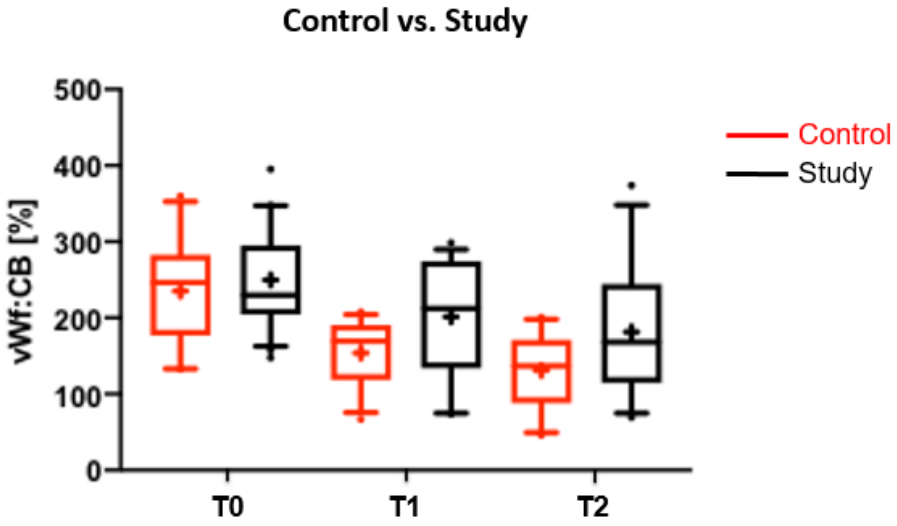


Figure 4.2 – Boxplot representing the distribution of vWf:CB activity in the two groups (control vs. study) at time points T0, T1, and T2. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

No significant differences were observed between the two groups at each of the considered time points:

- T0: vWf:CB (control) = 246.4% (177% - 282.8%); vWf:CB (study) = 229.7% (204.6% - 295%) (p-value 0.58);
- T1: vWf:CB (control) = 169.4% (118.4% - 190.6%); vWf:CB (study) = 212.5% (134.1% - 274.4%) (p-value 0.07);
- T2: vWf:CB (control) = 137% (88.2% - 171.1%); vWf:CB (study) = 167.9% (114.8% - 244.3%) (p-value 0.12).

Then, the study population was evaluated before and after ASA suspension to compare vWf:CB activity with and without ASA. Experimental data (Figure 4.3) demonstrate no statistically significant changes in the functionality of the vWf after ASA suspension (p-value 0.72). In detail: vWf:CB (w ASA) = 190.8% (114.8% - 264.1%); vWf:CB (w/o ASA) = 218.6% (121.4% - 261.4%).

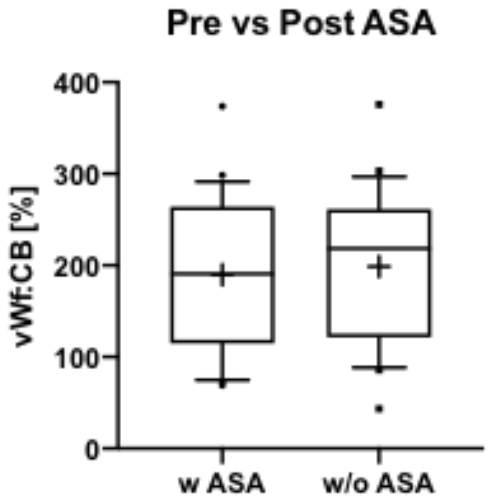


Figure 4.3 – Boxplot of vWf:CB activity in the study group before and after ASA suspension. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

Moreover, the comparison between the different pumps implanted was performed. Data from HMII pump were not considered due to low sample numbers (n=2). Results showing vWf:CB activity related to HM3 and HVAD over time are reported in Figure 4.4.

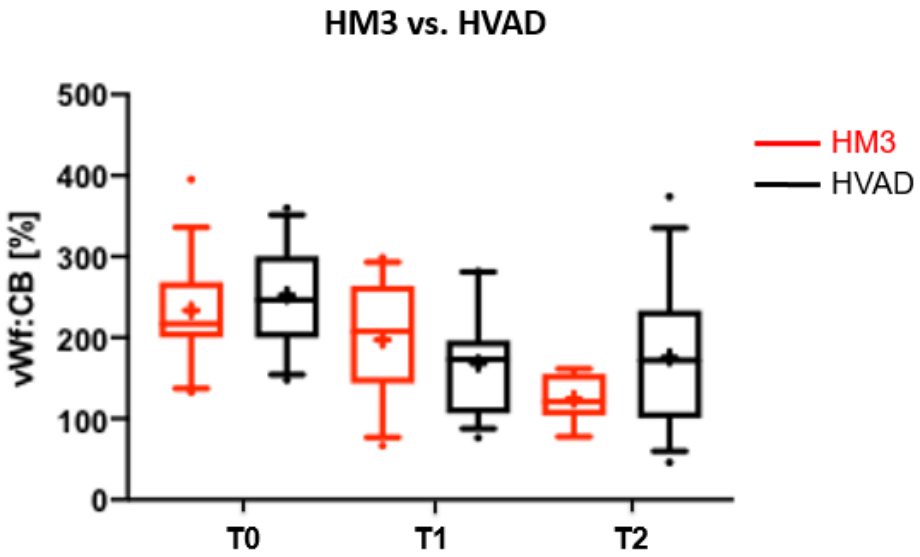


Figure 4.4 - Comparison of vWf:CB activity values according to the pump models of patients enrolled in the present study. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

Median vWf:CB values are the following:

- T0: vWf:CB (HM3) = 216.7% (200.3% - 273.6%); vWf:CB (HVAD) = 246.6% (199.7% - 300.7%) (p-value 0.41);
- T1: vWf:CB (HM3) = 207.9% (143.3% - 264.1%); vWf:CB (HVAD) = 172.9% (106.3% - 196.6%) (p-value 0.27);
- T2: vWf:CB (HM3) = 113.4% (77.8% - 200.5%); vWf:CB (HVAD) = 167.9% (105.4% - 228.5%) (p-value 0.34).

No significant changes according to the pump model used are observed. However, the two pumps act differently on the degradation profile: at T1 vWf is subjected to a slower degradation with HM3; conversely, at T2 the factor is further degraded by HM3 whereas its activity to bind collagen remains almost unchanged with HVAD.

In conclusion, the results of these analyzes suggest that:

i. Considering the overall population, the ability of von Willebrand factor to bind collagen is significantly reduced already in the early post-implant. This finding agrees with literature, stating that patients with an LVAD develop a consistent profile of reduced HMW vWf multimers already in the first weeks of device support. HMW

multimers are responsible for binding collagen, therefore, their reduction translates in a functional deficit of the factor.

ii. Comparing the two groups, values of vWf:CB do not change significantly, meaning that patients who suffered a bleeding event were not characterized by a different, more severe, degradation profile of the vWf. This aspect may suggest that the analysis alone of vWf activity is not sufficient to stratify patients at higher risk of developing a bleeding event.

iii. Regarding the study group, the comparison of vWf:CB values pre- and post- ASA suspension indicates there are no significant changes. This finding suggests that patients of the study group who did not develop a further bleeding event were not characterized by any restoration of vWf activity; conversely, positive clinical outcomes might be associated with the positive effects induced by ASA suspension.

iv. Making a comparison between different pump types, both devices contribute to the progressive degradation of vWf with no significant difference in terms of action severity of the pump. Considering vWf:CB values, at T1 a milder effect on the degradation profile can be observed with HM3 rather than with HVAD; conversely, in the long-term follow up (T2) HM3 has a further negative contribution on vWf whereas vWf:CB values remain almost constant with HVAD.

4.4 Analysis of prothrombotic platelet activity

Patients enrolled in the present study were subjected to the analysis of their thrombotic profile by considering: (i) coagulation parameters and (ii) platelet activation measured via PAS assay.

Same considerations made for the analysis of vWf activity, regarding hematochemical parameters and considered time points, were assumed (Table 4.2).

PAS values of the overall population are reported in Figure 4.5.

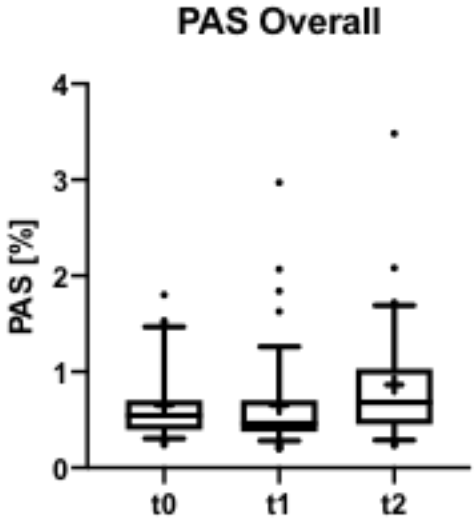


Figure 4.5 – Boxplot of PAS values distribution in the overall population at T0, T1 and T2. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

Results do not show a statistically significant variation of PAS value over time (p-value 0.42). Median PAS values recorded are the following: PAS (T0) = 0.56% (0.40% - 0.72%); PAS (T1) = 0.46% (0.38% - 0.71%); PAS (T2) = 0.68% (0.45% - 1.03%). However, PAS increasing trend agrees with literature: platelet activation shows a dose and time dependency to shear stress exposure.

After that, a comparison between the two groups (control vs. study) was done. Data reported in Figure 4.6 reveal no statistical significance among the two groups at each time point considered.

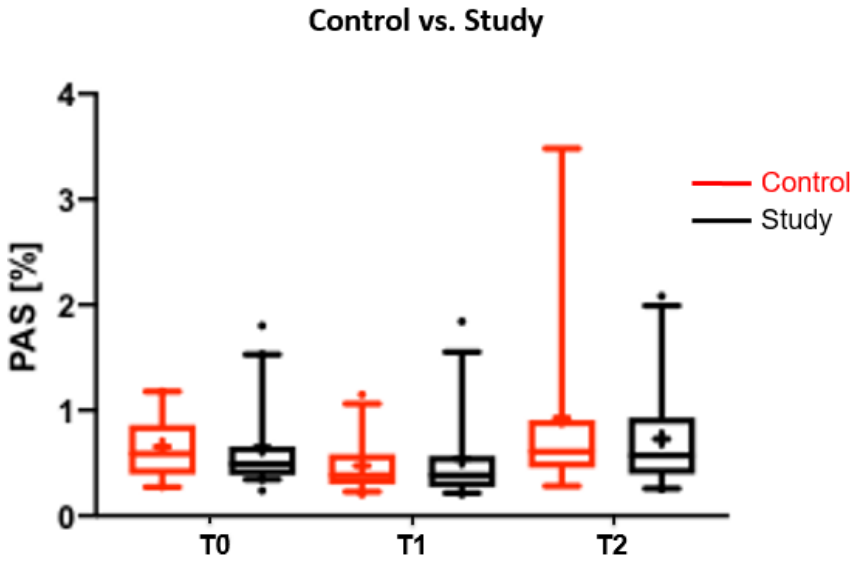


Figure 4.6 – Boxplot representing the comparison of PAS values between the two groups at T0, T1 and T2. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

The comparison shows there are no significant variations among control and study group over time. Considering the different time points, median values are:

- T0: PAS (control) = 0.59% (0.39% - 0.86%); PAS (study) = 0.49% (0.39%-0.68%) (p-value 0.52);
- T1: PAS (control) = 0.39% (0.30% - 0.59%); PAS (study) = 0.39% (0.27% - 0.57%) (p-value 0.67);
- T2: PAS (control) = 0.61% (0.46% - 0.91%); PAS (study) = 0.57% (0.40% - 0.93%) (p-value 0.59).

Moreover, PAS values representing PA profile of the study group before and after ASA suspension were analyzed (Figure 4.7). Statistical analysis showed there is a significant difference between the two conditions regarding administration of ASA (p-value 0.002).

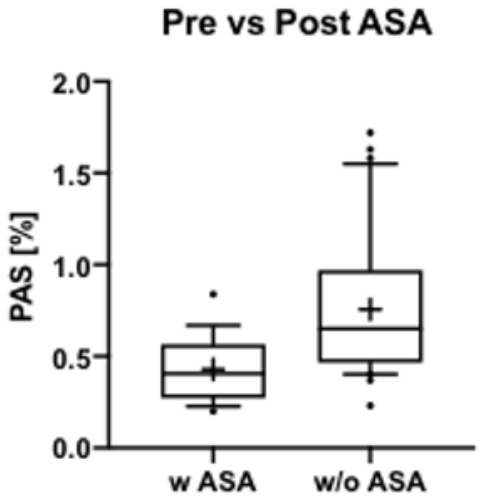


Figure 4.7 – Boxplot of PAS values before and after Aspirin suspension in the study group. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

In detail: PAS (w ASA) = 0.45% (0.30% - 0.70%); PAS (w/o ASA) = 0.68% (0.48% - 1.05%). Median value of PAS without ASA is 1.5 times higher than with ASA, showing the sensitivity of PAS assay with respect to antithrombotic regimen of patients. The only patient who developed a thrombotic event had, however, a mean PAS value higher than the patients of the same group (1.15 vs. 0.82); this aspect suggests the patient was probably more inclined to develop a thrombotic event. Considering the rest of the patients, it is reasonable to expect higher values of platelet activation in patients who suspended AT therapy.

A sub-analysis of PAS values according to different pump models was also made. Figure 4.8 depicts data measured in patients supported with the HM3 or the HVAD.

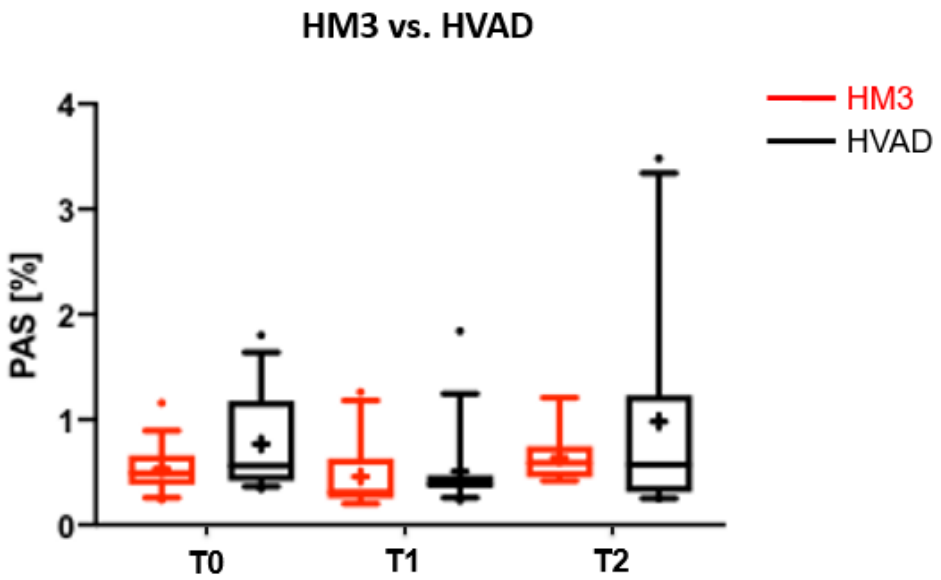


Figure 4.8 – Comparison of PAS values according to the pump models of patients enrolled in the present study. Bars indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles; + indicates mean values; dots indicate outliers.

Results show no significant differences in PA induced by the two devices at the considered time points. In detail:

- T0: PAS (HM3) = 0.49% (0.38% - 0.66%); PAS (HVAD) = 0.56% (0.42% - 1.18%) (p-value 0.10);
- T1: PAS (HM3) = 0.38% (0.27% - 0.55%); PAS (HVAD) = 0.39% (0.36% - 0.45%) (p-value 0.99);
- T2: PAS (HM3) = 0.59% (0.45% - 0.75%); PAS (HVAD) = 0.73% (0.48% - 0.99%) (p-value 0.13).

In conclusion, these analyzes suggest that:

i. Overall, PAS values do not show any significant difference over time. It can be observed that at T2 patients have an increase in platelet activation level, even if not statistically significant; this aspect agrees with literature confirming the progressive effect of shear stress on PA.

ii. Patients who suspended ASA showed a significant increase of PA in terms of PAS values. Despite these values do not indicate a high thrombotic risk (as it is confirmed by coagulation parameters and by the fact that only 1 patient effectively developed a

thrombotic event), they demonstrate the efficacy and sensitivity of PAS assay for both prothrombotic platelet activity and the effects of antithrombotic regimen.

iii. The difference between implanted pumps did not affect the patient's exposure to thrombotic risk. Indeed, PAS values do not significantly differ if comparing HM3 and HVAD platelet activation state, suggesting a concurrent patient-specific tendency to develop post-implant complications.

iv. Regarding patients who developed a thrombotic event, some considerations need to be done. The patient who suffered the event after ASA suspension had a mean PAS value higher than the rest of the patients of his own group, meaning that he had an higher platelet activation state compared to baseline. Similarly, 1 out of 2 patients of the control group had a mean PAS value higher (PAS values: 1.94 vs. 0.66) and an increment of 223% was registered before developing the thrombotic event. The second patient had, instead, a mean PAS value in the group range but, in the day of pump thrombosis development, PAS value was 128% higher than the previous one registered. These considerations underline the ability of PAS assay to predict the occurrence of a thrombotic event and its utility in clinical routine.

Chapter 5

Conclusions

The present study aimed at evaluating the competing role of von Willebrand factor disease, prothrombotic platelet activation, and antithrombotic pharmacological therapy in exposing CF-LVAD patients to the development of HRAEs (hemorrhagic and thromboembolic events) over the time of LVAD support.

Several studies demonstrated how supraphysiological shear stress imposed by the pump lead to: (i) a progressive degradation of von Willebrand factor high-molecular-weight multimers, which reflects on a deficit of its functional activity; (ii) a triggering effect on platelet activation. These factors expose the patient, respectively, to bleeding and thrombotic risks.

Despite antiplatelet therapy (ASA) is commonly prescribed in clinical practice to prevent the occurrence of thromboembolic events, recent studies indicated ASA as a possible cause of the development of bleeding complications. In parallel, it has been suggested that ASA does not have a primary role in preventing thrombotic risk in the setting of LVAD support; moreover, its suspension does not increase the incidence of such events but determines a reduction of related hemorrhagic events.

On the other hand, the combined action of these three factors and how they affect the hemostatic profile of LVAD patients, to date, was poorly analyzed.

On this background, the analysis performed suggests there is a patient-specific tendency (related to a specific clinical status) in developing hemorrhagic events, which, however, can be limited by the suspension of ASA. On the other hand, this suspension does not lead to a higher risk of developing thrombotic events.

At this purpose, tested population was divided in patients who suffered a bleeding event (study) and patients who did not (control).
Results from vWf collagen binding activity assay demonstrated that in all patients the factor is subjected to degradation after device implant, without any difference between the two groups. Moreover, in patients who developed hemorrhagic events, this loss of functionality does not recover after ASA suspension. Firstly, these data propose that the analysis of von Willebrand factor degradation profile alone is not sufficient to discriminate patients on the basis of their tendency to develop a bleeding event; secondly, they highlight how the suspension of antiplatelet regimen has a positive effect, for the 81% of tested population, in preventing a further bleeding event since vWf degradation profile remains almost unchanged before and after the suspension of the drug.

In parallel, the current work analyzed the effect of ASA suspension on prothrombotic platelet activity. Despite PAS results showed a more activated state of platelets after the suspension of the drug, demonstrating the sensitivity of PAS assay to antiplatelet therapy, only 1 patient developed a thrombotic event who had already a higher PAS value if compared to baseline PAS of his own group. This aspect agrees to what said before: the detrimental effect of shear stress does not seem to be mitigated by ASA.

Furthermore, the comparison between HM3 and HVAD showed no differences both on vWf degradation profile and platelet activation. In addition, patients supported with the same pump were not subjected to the same probability to suffer an adverse event. This observations lead to the conclusion that the pump is not the only determinant of events triggering but other factors, as patient-specific tendency, surgery-related factors, and patient response to pharmacological therapy, may have a role in the development of hemorrhagic and thromboembolic events.

In the light of these findings, the present thesis integrated the analysis of the possible hemorrhagic causes (vWf degradation) to the study of PA level and AT efficacy which, conversely, are related to the thrombotic risk. This aspect allows to obtain a wider and more detailed picture of the clinical condition of these patients.

In conclusion, the current work suggests that a variation in the antithrombotic pharmacological therapy may contribute to reduce the incidence of hemorrhagic events in the setting of LVAD implantation and, in parallel, does not significantly increase the thrombotic risk. Moreover, it highlights the need of more patient-specific pharmacological strategies that, coupled with a constant monitoring of platelet activation state and clinical status of the patients, could preserve the delicate balance within the two arms of coagulation complications (hemorrhagic and thromboembolic).

5.1 Study limitation and future implications

The present study is a single-center study with a relatively small number of patients (n = 45) and hemorrhagic events registered (n = 26).

Further multicentric studies are warranted to allow the enrollment of a larger patient cohort and comparative trials. This will permit to evaluate the efficacy and safety of a reduced antithrombotic therapy as a strategy to reduce the occurrence of bleeding events without increasing the thrombotic risk. Moreover, it will help in the validation of results by excluding that the increase of sample size may reveal higher thrombotic risk developed by patients not on ASA.

A possible future implication could be the investigation of the interaction between ASA and LVAD patients from the bleeding point of view. Indeed, the analysis on vWf degradation profile demonstrated that the factor, after ASA suspension, does not recover from degradation remaining unchanged and, at the same time, 81% of patients stopped bleeding. Consequently, ASA could be the variable responsible for this shift in patient's response. Starting from this consideration, further investigations are needed in order to understand why some patients are more exposed to bleeding events than other patients while on ASA. In clinical practice, a patient-specific characterization may include: (i) improved selection of the patients who should receive or not AT therapy; (ii) ongoing review of when it is safe to

discontinue the therapy; (iii) the use of laboratory techniques which can determine adequacy of inhibition of platelet aggregation.

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