

Shear-Mediated Platelet Activation in Patients Implanted with Continuous Flow LVADs: a Preliminary Study Utilizing the Platelet Activity State (PAS) Assay*

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Abstract – Left ventricular assist devices (LVADs) have emerged as vital life-saving therapeutic systems for patients with advanced and end-stage heart failure (HF). Despite their efficacy, VAD systems remain limited by post-implantation thrombotic complications. Shear-mediated platelet activation is the major driver of such complications in these devices.

Nowadays few platelet function assays are routinely utilized in assessing the degree of platelet activation in VAD implanted patients. No assays exist that specifically target shear-mediated platelet activation. The platelet activity state (PAS) is a novel assay that has been well validated *in vitro*, measuring thrombin release as a surrogate for shear-mediated platelet activation. To date limited data exist as to the utility of this assay in the clinical setting. In the present study we evaluated eight LVAD patients' platelet activation level using the PAS assay. Simultaneous measurements of conventional prothrombotic and hemolysis markers, - i.e. fibrinogen and lactate dehydrogenase (LDH) - were also performed. Trends as to alteration from baseline were studied.

We observed that the PAS assay allowed detection of an abnormal level of platelet activation in one patient in our series who suffered from an overt thrombosis. Interestingly in the same patient no signal of major abnormality in fibrinogen or LDH was detected. Further for 7/8 patients who were free of thrombosis, no significant level of platelet activation was

detected via PAS assay, while elevation in fibrinogen and LDH were observed. As such, from our observational series it appears that the PAS assay is a sensitive and specific indicator of shear-mediated platelet activation. Further patients' experience will help elucidate the role of this promising assay in the management of LVAD implanted patients.

I. INTRODUCTION

Ventricular assist devices (VADs), while effective in hemodynamic restoration for patients with advanced heart failure (HF), remain burdened with several post-implant complications including pump failure, infection, hemolysis or thrombotic events [1-3]. Hemolysis and thrombus formation are primarily due to the high level of shear stress imparted to blood in repeated passage through these devices. Indeed, increased shear stress is a hallmark of flow conditions in blood-recirculating devices [4].

Research studies to date, examining the impact of cardiac devices on platelet activation and thrombosis led to the development of the platelet activity state (PAS) assay [5]. Utilizing a modified prothrombin precursor reagent, i.e. acetylated prothrombin, it was demonstrated that shear-mediated platelet activation would lead to conversion of prothrombin to thrombin, but without the feedback of further platelet activation or conversion of fibrinogen to fibrin. As such a stoichiometric relationship is defined allowing detection of thrombin to serve as a clean indicator of shear-mediated platelet activation. This assay has been utilized extensively for *in vitro* studies of device-associated platelet activation, particular characterizing devices and situation with a wide range of imposed shear stress [4, 6, 7]. Despite this body of *in vitro* knowledge, limited data exist as to the utility of the PAS assay to be employed in the clinical setting. In the present study we examine the use of the PAS assay as a means of detecting altered platelet activity in VAD-implanted patients [8].

Presently, several parameters are utilized clinically as laboratory markers of VAD-related thrombosis or hemolysis. Along with standard blood parameters (hemoglobin, platelet count) and coagulation markers as prothrombin time (PT), partial thromboplastin time (PTT) and the international normalized ratio (INR), other elements such as lactate dehydrogenase (LDH) or fibrinogen blood concentrations are typically tracked, as markers of hemolysis and prothrombosis, respectively [9].

*Research supported by Consorzio Interuniversitario per la Scienza e la Tecnologia dei Materiali (INSTM) and San Raffaele Hospital (project title "Studio di superfici bioattive per l'ottimizzazione degli LVAD (Left Ventricular Assist Device): Bioactive Surfaces for Optimized LVAD"), and by 2011-2241 CARIPO Foundation grant (project title "Evaluation of the thrombogenic risk: development of new approaches for the prediction of platelet activation and its minimization in artificial organs and prosthesis").

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In the present study we have assayed the platelet activity state of eight LVAD-implanted patients along with measurement of fibrinogen and LDH.

Although preliminary, this study represents a first attempt to assess the reliability of using the PAS assay directly in the clinical setting to discriminate major clinical issues with regard to VAD-related thrombosis.

II. MATERIALS AND METHODS

A. Subjects

Eight patients with continuous LVAD (four Thoratec HMII VAD – four Heartware HVADs) (mean age 64.5 +/- 12.5 years, days after implant 45 +/- 39) were involved in the study. All subjects underwent standard medication regimen after implant, consisting in warfarin dosed to maintain INR target level of 2-2.5 and acetylsalicylic acid (ASA) in a concentration range of 75-300 mg/day.

Among all subjects, one patient - Patient 1, developed VAD thrombosis and underwent pump replacement 14 days prior PAS assay analysis. PAS analysis, as well as LDH and fibrinogen levels detection, were performed after LVAD implantation for all subjects. The protocol was approved by the Institution's Ethic Committee and a written informed consent to the study was obtained from each subject.

The overall experimental design is schematically shown in Fig 1.

B. Platelet preparation

Whole blood (20 ml) was drawn via venipuncture into 3 ml acid-citrate dextrose (ACD-A) from consenting LVAD patients. Blood was centrifuged at 500g for 15 min to obtain platelet-rich plasma (PRP), which was gel-filtered through a column of Sepharose 2B beads (GE-Healthcare Life Sciences) to collect gel-filtered platelets (GFP) [7].

GFP were diluted to a count of 20,000 platelet/ μ l in HEPES-modified Tyrode's buffer, with 3 mM $CaCl_2$ added 10 min prior to experiments [10-11]. At this point, platelet activation level was detected via PAS assay.

C. Platelet activity state (PAS) assay

The Platelet activity state (PAS) was measured using the modified-prothrombinase assay [5]. This method utilizes a novel stoichiometric relationship identified in a clinical coagulopathy known as "Prothrombin Quick disorder". Although thrombin generation is a powerful marker of platelet activation in response to several biochemical and mechanical agonists, the produced thrombin has a positive feedback response on platelet activation. To block thrombin feedback and ensure a one-to-one relation between the inducer and the measured platelet activity level, acetylated-prothrombin (Ac-FIIa) is used as the thrombin substrate. Ac-FIIa does not feedback on the factor Xa complex to further activate platelets or convert fibrinogen to fibrin (Fig.1). The removal of the positive feedback activation by thrombin is essential for reliable quantification of platelet activation level. Thrombin generation was quantified through spectrophotometric analysis over 8 min at an absorbance wavelength of 405 nM, using Chromozym-TH (Roche Life Science) as the thrombin-specific chromogenic peptide substrate [7]. Linear regression analysis was performed on the changes in absorbance/min (the thrombin generation rate, i.e. the PAS value) and the value normalized against the thrombin generation rate of fully activated platelets, obtained by sonication (10 W for 10 s, Misonix Microson ultrasonic cell disruption) of platelet preparations. Accordingly, all PAS values were expressed as a percentage of the maximum thrombin-generating capacity, with a maximum of 100%.

D. Data Analysis

PAS assay measurements were performed on GFP from patients (one sample for each subject) obtained 45 +/- 39 days after implant. Measurements were repeated eight times for each subject. Non-parametric (Kruskal-Wallis) one-way ANOVA was performed to compare the PAS values obtained for the different subjects. Data obtained from PAS assay were also compared to LDH and fibrinogen values

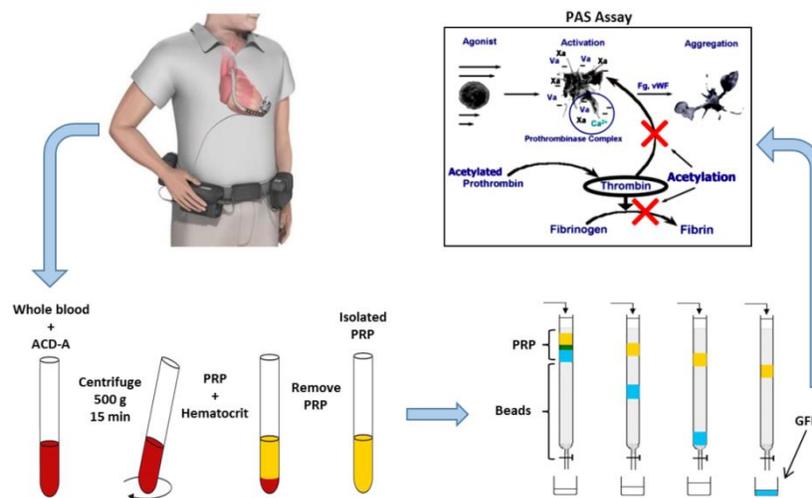


Figure 1. Overall experimental design. VAD-patients, PRP were extracted from whole blood after centrifugation. GFP were obtained through gel chromatography and platelet activation level assessed using the PAS assay, after addition of reagents for the measurement of the platelet-released thrombin.

measured from the same blood sample. Physiological range values for LDH and fibrinogen concentration in blood of healthy subjects were considered 125-220 U/L and 150-400 mg/dl, respectively.

III. RESULTS

The PAS assay level, LDH and fibrinogen concentrations obtained for the eight LVAD patients involved in the study are reported in Fig.2.

The platelet activity level detected for 7/8 patients (Pts 2 - 8) was less than 1%, thus indicating a minimal level of shear-mediated platelet activation in these subjects. In contrast for Patient 1, who sustained LVAD associated thrombus formation, a significant increase in PAS was detected compared to the other seven patients who remained thrombus free (15.5+/- 1.9 % vs 0.5 +/- 0.3 %, $p < 0.05$).

In contrast, LDH and fibrinogen concentrations detected in all subjects were determined to be elevated with respect to physiological values (except for fibrinogen level in Patient 6, see Fig. 2). No correlation was observed between the PAS and the level of fibrinogen and LDH. It is worth noting that the LDH and fibrinogen concentrations were not able to

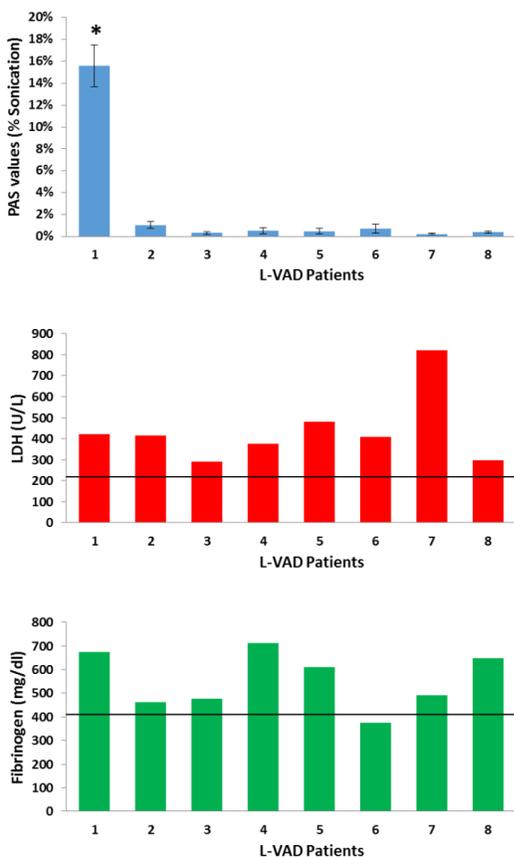


Figure 2. PAS assay, LDH and fibrinogen values obtained for eight LVAD patients (1-8). PAS values are reported as mean +/- standard deviation (number of replicates, n=8). * indicates statistically significant difference ($p < 0.05$). The Student–Newman–Keuls *post hoc* test was performed for comparisons between individual groups, after one-way ANOVA. The upper physiological limit (black lines in the graphs) of LDH and fibrinogen concentrations are considered 220 U/L and 400 mg/dl, respectively.

discriminate between patients plagued by thrombotic events (Patient 1) and the other subjects.

IV. DISCUSSION

In this study we report a clinical investigation in which we evaluate LVAD patient's platelet activation level via the PAS assay. Several studies in the literature support the ability of this methodology for assessing VAD-associated shear-mediated platelet activation *in vitro* [4,7]. To date the role of the PAS assay for use in the clinic has not been explored. We compared PAS values with standard clinical diagnostic tools (fibrinogen and LDH blood concentrations). To the best of our knowledge, no direct comparison among PAS assay, LDH and fibrinogen levels has been undertaken so far.

LDH and fibrinogen are considered of a pivotal interest to monitor phenomenon such as VAD-related hemolysis and thrombosis. In fact, in the literature, implantation of VADs has been associated with hyperfibrinogenemia, with circulating fibrinogen concentrations up to 400–900 mg/dl [12]. Patients with VADs and hyperfibrinogenemia are considered at increased risk of forming faster growing, stronger clots that dissolve more slowly than clots formed with physiologic fibrinogen concentrations. We observed similar pathological fibrinogen concentrations (Fig. 2) in all the patients, with a peak value of 712 mg/dl for Patient 4.

Nonetheless, fibrinogen levels usually increase early after LVAD implantation and return to baseline around 6–12 months post-implant [13]. For this reason, the fibrinogen level is not routinely screened in LVAD patients, especially in the post-implant phase. In fact, confirming thrombosis activity using fibrinogen levels early after device placement can lead to misinterpretation (because of initial protein absorption by device blood contact surface and active fibrinolysis).

In our settings, LVAD patients were monitored within the early post-implant phase (45 +/- 39 days), thus leading to increased fibrinogen levels that could be misleading for a possible thrombosis diagnosis. However, a continued increase in fibrinogen levels or new evidence of its increase after returning to baseline may indicate ongoing thrombosis and/or fibrinolysis activity and warrant further investigation. In a next phase of the study, continuous and long-term monitoring of the patients after implant would allow us to identify possible further correlations among coagulation markers as fibrinogen and the risk of thrombogenesis.

Thrombus formation in the pump can also trigger hemolysis. The latter remains a clinically relevant adverse effect associated to several VAD technologies. LDH and hemoglobin are prominent factors released by lysed erythrocytes [9]. Accordingly, LDH level may independently corroborate an increasing incidence of pump thrombosis. In particular, the occurrence of elevated LDH levels within 3 months after implantation may indicate thrombus formation. In a study presented by Starling and colleagues, thrombosis was presaged by LDH levels that increased from 540 U/L to 1490 U/L within the weeks before diagnosis [14]. Sudden increase in baseline LDH

levels may thus suggest further investigations based on clinical evidence in the form of imaging studies and thrombosis-specific laboratory tests to rule out thrombosis activity.

Although we did not registered such increased LDH levels in our experiments, these studies underline that thrombosis activity, if diagnosed early, can prevent fatal outcomes and reoperation for device explant/substitution in these patients [14].

PAS assay analysis conducted in this study showed an interesting trend. Among all patients tested, only Patient 1 presented an elevated baseline level of platelet activation (15.5 +/- 1.9 % compared to sonicated platelets). Patient 1 had a clinical history of pump thrombosis and underwent removal of the clotted device and placement of a new LVAD 14 days prior to PAS assay measurements. Despite the occurrence of his clinical thrombotic event, LDH and fibrinogen levels detected for Patient 1 did not differ substantially with respect to the other patients involved in the study, thus indicating a lower sensitivity offered by these diagnostic parameters.

Although data obtained with PAS assay are promising, the present study highlights several limitations. First, we only analyzed a small number of L-VAD patients. Moreover, we were not able to record the level of platelet activation, LDH and fibrinogen pre-implant or for long time periods after implant, but only for a single time point for each patient. In addition, results obtained early after VAD implantation may be affected by acute reactions due to the surgical procedure and multiple transfusions, a factor that must be taken into account for correctly interpreting our results. In future studies, we further plan to consider the effect of anticoagulant and antithrombotic treatments on PAS, which may affect the results as well. Since in our study the drug therapies were similar among patients, their effects on the comparison should be negligible.

Nonetheless, results obtained to date demonstrated that the PAS assay detected abnormal levels of platelet activation in a patient who suffered from an overt thrombosis. Moreover, the assay demonstrated that platelets don't recover immediately after pump substitution, thus suggesting that monitoring possible clot rebuilt is essential to prevent the recurrence of thrombosis. Further investigations should aim to extend both the population of patients and the duration of the monitoring, thus allowing to clearly verify the reliability of PAS assay to spot VAD-related abnormal hematic conditions in the clinical setting.

V. CONCLUSION

Among studies conducted with the PAS assay to evaluate VAD-related thrombotic complications, our study represents the first attempt to connect such methodology to clinically relevant diagnostics. The PAS assay appeared to be a sensitive tool for detecting abnormal levels of platelet activation in association with VAD-related thrombosis compared to LDH and fibrinogen measurements. Nonetheless, these observations need to be supported by a larger pool of patients to further correlate PAS values with

diagnostic data routinely used clinically. This would allow a better evaluation of the potential reliability of the PAS assay to be established as a relevant clinical diagnostic tool to assess the thrombogenic potential in VAD-recipients. This type of investigation may potentially pave the way for further research studies aimed at realizing a new-generation of diagnostic devices for monitoring VAD patients' health conditions. These devices will reduce the likelihood of thromboembolic events, providing better clinical and therapeutic solutions improving the quality of life of VAD recipients, and significantly reducing the healthcare burden, allowing shorter hospitalizations and limiting post-implantation complication rates and pharmacological therapy costs.

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