Published in final edited form as:

Thromb Res. 2017 October ; 158: 140–148. doi:10.1016/j.thromres.2017.09.006.

Oxidative stress induced modulation of platelet Integrin $\alpha 2b\beta 3$ expression and shedding may predict the risk of major bleeding in heart failure patients supported by continuous flow left ventricular assist devices

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Abstract

Introduction—Oxidative stress and platelet integrin $\alpha 2b\beta 3$ plays important role in the process of hemostasis and thrombosis. We hypothesized that device-induced patient specific oxidative stress and integrin $\alpha 2b\beta 3$ shedding may be linked to major bleeding complication (MBC) in heart failure (HF) patients supported by continuous flow left ventricular assist devices (CF-LVADs).

Materials and Methods—We recruited 47 patients implanted with CF-LVADs and 15 healthy volunteers. Fourteen patients developed MBC (bleeder group) within one month after implantation while others were considered non-bleeder group (n=33). Oxidative stresses were evaluated by measuring reactive oxygen species (ROS) in platelets, superoxide dismutase (SOD) activity, total antioxidant capacity (TAC) and oxidized low density lipoprotein (oxLDL). Assessments of $\alpha 2b\beta 3$ were carried out using flow cytometry and ELISA.

Results—Biomarkers of oxidative stress and $\alpha 2b\beta 3$ shedding (decreased surface expression and higher plasma levels) were found to be preexisting condition in all HF patients prior to CF-LVAD implantation compared to the healthy volunteers. Significantly elevated levels of ROS and oxLDL; concomitant depletion of SOD and TAC; and $\alpha 2b\beta 3$ shedding were observed in the bleeder group temporarily in comparison to the non-bleeder group after CF-LVAD implantation. A significantly

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Conflicts of interest: None

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strong association between $\alpha 2b\beta 3$ shedding and biomarkers of oxidative stress was observed; suggesting a potential role of oxidative stress in platelet integrin shedding leading to MBC after CF-LVAD implantation. Moreover, a receiver operating characteristic (ROC) analysis indicated that the likelihood of MBC data from Integrin $\alpha 2b\beta 3$ shedding had a predictive power of MBC in CF-LVAD patients.

Conclusions—Oxidative stress might play a potential role in accelerating $\alpha 2b\beta 3$ shedding and platelet dysfunction, resulting in MBC in CF-LVAD patients. Integrin $\alpha 2b\beta 3$ shedding may be used to refine bleeding risk stratification in CF-LVAD patients.

Keywords

Heart failure; CF-LVAD; major bleeding; oxidative stress; platelet Integrin $\alpha 2b\beta 3$

Introduction

Heart failure (HF) is the leading cause of death in the US affecting more than 5 million Americans. Despite medical advances and an annual expenditure of \$34.4 billion in the US, 50% of diagnosed HF patients will succumb within 5 years [1,2]. Mechanical circulatory support (MCS) therapy has evolved into a standard therapy for patients with advanced HF [3,4] either as destination therapy, or as bridge to cardiac transplant, or as a platform for myocardial remodeling/recovery [5,6]. Advances in CF-LVADs have also demonstrated improved device reliability, durability, and performance. The number of HF patients receiving life-saving CF LVAD therapy has quadrupled over the last five years [7] with 6-month (90%) and 1-year (85%) survival rates [8,9]. However, device-related adverse events (bleeding, infection, stroke) as well as right ventricular failure and multi-organ failure remain significantly clinical problems [10–13]. The annual report from the Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) showed that major bleeding complication (MBC) is the most common post-operative complication in HF patients supported by CF-VADs [7].

Platelets are critical for the maintenance of the integrity of the vascular system and are the first line of defense against hemorrhage [14]. Platelet dysfunction is an important cause of major bleeding after cardiac surgery [15]. Recently, it was reported that platelet glycoprotein Iba ($GPIb\alpha$) ectodomain shedding may be linked to major bleeding in CF-LAVD patients [16]. Non-physiological high shear stress has been suggested to contribute to the shedding of key platelet receptors [17–19]. Studies also reported that oxidative stress may also influence the alteration of platelet and leukocyte functionality in CF-LAVD patients with systemic inflammation, infection and sepsis [20,21]. Oxidative stress and peroxide synthesized in the platelet lipoxygenase pathway activates tumor necrosis factor- α -converting enzyme and induce shedding of key platelet receptors in murine and human model [22]. Moreover, other study reported that serum total oxidative stress levels were higher and serum total antioxidant capacity levels were lower in pregnant women who had higher vaginal bleeding when compared to women with normal-ongoing pregnancies of similar gestational age in the first trimester [23]. Thus higher oxidative stress may have a role in mediating abnormal bleeding [23].

Upon activation, platelets change shape, release granules, and firmly adhere and aggregate through activation of the adhesive receptor integrin $\alpha 2\beta 3$ [24]. The regulation of platelet function is finely tuned by a balance between the vasculature's redox environment and the oxidative processes that occur in it [25]. Thus the importance of redox-regulation of integrin $\alpha 2\beta 3$ functionality cannot be underestimated. It appeared that in extremely rare coagulopathy platelets contain defective or low levels of integrin $\alpha 2\beta 3$; as a result the bleeding time is significantly prolonged [26]. Elevated levels of oxidative stress are common in HF patients [27–29]. Further increased levels of oxidative stress been reported in platelets from some HF patients supported with CF-LVAD in our previous report and associated with bleeding in CF-LVAD patients [30]. The elevation of oxidative stress in platelets of HF patients after CF-LVAD implantation and its association with platelet integrin $\alpha 2\beta 3$ abnormalities and MBC have not been well examined. Unfavorable response to CF-LVAD implantation, leading to increased production of oxidative stress, in combination with non-physiological mechanical factors could induce defect in platelet integrin $\alpha 2\beta 3$. The present study focused on the status of oxidative stress and integrin $\alpha 2\beta 3$ expression, shedding and activation which may be linked to MBC in CF-LVAD patients.

Materials and Methods

Study design and sample size

In this prospective observational study, we studied 47 HF patients with NYHA class IV undergoing CF-LVAD implantation as bridge to transplant or destination therapy and 15 healthy volunteers as the reference group. The CF-LVAD patients were divided into two groups: (i) bleeder group (n=14; MBC within one-month after device implantation and (ii) non-bleeder group (n=33). MBC was defined according to INTERMACS classification. CF-LVAD patients having bleeding that requires a transfusion of 4 or more units of packed red blood cells and/or causes hemodynamic instability requiring inotropic infusion and/or surgical reintervention was considered bleeders. Twenty one patients received HeartMate II CF-LVAD (Thoratec Corp, Pleasanton, CA), eleven patients-the Jarvik 2000 CF-LVAD (Jarvik Heart, New York, NY), and fifteen patients-the HeartWare HVAD LVAD (HeartWare Inc, Framingham, MA). Sample size calculation and power analysis were carried out in sample size software PASS (version 14.0.7, NCSS, LLC, Kaysville, Utah, USA) followed by the formula of Julious 2010 [31]. Group sample sizes of 33 (non-bleeder) and 14 (bleeder) achieve 85.315% power with a significance level (alpha) of 0.050 using a two-sided two-sample unequal-variance t-test. All procedures involving collection of human blood were approved by the Institutional Review Boards (IRB). All patients and volunteers gave their written informed consent and were informed about the aims of the study in accordance with the Declaration of Helsinki.

Inclusions and exclusions criteria

The inclusion criteria for the selection of HF patients were: (i) ages between 18 to 70 years, (ii) undergoing CF-LVAD implantation and (iii) able to provide informed consent for the study. The inclusion criteria for the control subjects were (i) no clinical evidence of HF or other cardiovascular diseases, (ii) no history of malignancy and (iii) no inflammatory disease

based on routine laboratory tests. Pregnant or breastfeeding women or women using oral contraceptives were also excluded from the study.

Anticoagulation/antiplatelet treatment

All the CF-LVAD patients in the study were treated with our standard anticoagulation and antiplatelet regimen. Anticoagulation was initiated with a titrated heparin dose with the goal for partial thromboplastin time of 40–45s once chest drainage was less than 30 mL/h for at least 4 hours. Thereafter the goal was aimed to have an anti-Xa activity level of 0.1–0.15 U/mL. The anticoagulation medication was subsequently converted to warfarin with a targeted international normalized ratio (INR) for the HeartMate II (1.8–2.3), the Jarvik 2000 (2.0–3.0) and the HeartWare HVAD (2.0–3.0). Antiplatelet agents were added to the anticoagulation regimen and the dosage was titrated based on measurements of platelet function using a platelet function analyzer (PFA-100®, Dade Behring, Inc, Deerfield, IL) and thrombelastogram (TEG) (TEG® 5000 Thrombelastograph® Hemostasis Analyzer System, Haemonetics Corporation, Braintree, MA). All the patients received pentoxifylline to improve red blood cell deformability in the hope of mitigating shear-induced hemolysis. Although individual patient anticoagulation/antiplatelet regimen might vary day-to-day, a standard strategy was followed for all the patients.

Collection and preparation of blood sample

EDTA/Citrate-anticoagulated blood samples from the HF patients were collected before CF-LVAD implant surgery (baseline/pre-operative: Pre-OP) and at day 7, 14, 21 and 30 (Post-operative duration 1, 2, 3 weeks and 1 month: POD-1W, 2W, 3W and 1M) after the implant surgery. A single blood sample was collected from each of the healthy donors. All HF patient and the healthy volunteer blood samples were aliquoted and processed immediately.

Assessment of oxidative stress

Different methods have been used for examining oxidative stress, each with potential pitfalls and advantages. All of these techniques may yield errors, and it is advisable to use two independent methods to avoid a mistaken conclusion [32]. In our study we measured 4 different biomarkers to examine oxidative stress status. In our present study, to detect oxidative stress, we used flow cytometry (FACS Calibur with sorter, Becton Dickinson, San Jose, CA, USA) to detect the generation of ROS (in terms of measuring mean fluorescence of DCF-DA dye) from platelets. As oxidative stress indicates an imbalance between antioxidant defenses and the production of ROS, we further measured the levels of potent antioxidant enzyme superoxide dismutase (SOD) and total antioxidant capacity (TAC) in plasma by using commercially available ELISA kits (SOD: Cell Technology, Inc., Mountain View, CA, USA; TAC: Randox Laboratories, Antrim, UK) to identify whether the antioxidant capacity was comprised or not. Finally, we also measured plasma levels of oxidized low density lipoproteins (oxLDL: another indicator of oxidative stress) using ELISA (Mercodia Inc, Winston Salem, NC) to confirm that the oxidative stress was a true observable fact in the present study. All the biomarkers and techniques used to measure oxidative stress are well established by us or others and details can be found in the literatures [20,25,26,30,32–35].

Assessment of platelet integrin $\alpha 2b\beta 3$

Flow cytometric assessment of platelet surface integrin $\alpha 2b\beta 3$ expression (CD41/61) and $\alpha 2b\beta 3$ activation (PAC-1) was performed by the well-established procedures [18,19]. All the monoclonal antibodies (FITC or PE conjugated) and their corresponding isotype controls were purchased from BioLegend, San Diego, CA (CD41, $\alpha 2b\beta 3$ -CD41/61, IgG2ak and IgG1k) and BD Biosciences, San Jose, CA (PAC-1, IgMk). These antibodies were raised against their respective antigens on the human platelet surface. In brief, for each experiment, 5 μ L of anticoagulated blood was diluted with 25 μ L HEPES (10 mM) buffer and incubated with 5 μ L (20 μ L for PAC-1) each of fluorescence isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies for 30 min at room temperature in dark. Thereafter the cells were fixed with 1.0% paraformaldehyde and kept at 4⁰C for 30 minutes, and 10,000 events were acquired and analyzed in a flow cytometer (FACS Calibur with sorter, BD, San Jose, CA, USA). To ascertain the sensitivity of the PAC-1 antibody, an agonist activated blood sample was used as a positive control. We constructed the positive control using healthy donor blood incubated with 20 microM adenosine diphosphate (ADP) and then with PAC-1 antibody. Platelets were gated on dot plots from their characteristic forward and side scatter (FSC and SSC respectively) and were identified as CD41 positive cells. Analyses of the acquired data were performed offline by using the FCS Express 4.0 software (De Novo Software, Los Angeles, CA). The expression of the platelet surface integrin $\alpha 2b\beta 3$ was expressed as mean fluorescence intensity (MFI). Our lab typically runs samples in duplicate or triplicate every time antibody lot changes, elaborate comparative staining is done in parallel to old lot. In case of baseline sample from each new patient, we run duplicate tubes. We run most of the follow-up experiment in single tube each for $\alpha 2b\beta 3$ and PAC-1. Flow cytometric assessment of platelet surface integrin $\alpha 2b\beta 3$ expression (CD41/61) and activation (PAC-1) were performed in freshly collected blood samples without stimulation of the platelets. To identify possible shedding of integrin $\alpha 2b\beta 3$ from the platelet surface to plasma, we also quantified the plasma level of $\alpha 2b\beta 3$ using a commercially available AssayMax human $\alpha 2b\beta 3$ ELISA kit (Assaypro LLC., Charles, MO) according to the manufacturer's instruction.

Statistical analyses

The data are presented as mean $\pm$ SD (standard deviation) or median with interquartile range (IQR). Statistical differences were determined by using Fisher's Exact Test (for results presented as percentages), Student's t-test (for results presented as mean $\pm$ SD), and Mann-Whitney 'U' test (for median values with IQR). One-way and repeated measures ANOVA with Tukey-Kramer adjustment for multiple comparisons were used for testing statistical differences between baseline and longitudinal data. Univariate analysis was carried out using Spearman's rank correlation test to find out the relation between two measurable parameters as continuous variables, and the result was expressed as rho value. Statistical significance was assigned at $p < 0.05$. For spearman's rank correlation analysis to identify any correlation between different biomarkers of oxidative stress and platelet integrin shedding, paired oxidative stress data for each biomarker from baseline to post operative day 30 in each patient plotted against the matched platelet receptor shedding data. The cumulative impact of biomarkers and potential baseline confounding factors (BMI) on the bleeding incidence was evaluated by multivariate logistic regression analysis. Apart from biomarkers, the

confounding factors were selected based on purposeful selection of variables i.e. any variables significantly different on univariate analysis. Any measured parameter was treated as a variable, either continuous (when computing univariately for correlation) or dichotomous (when examining association). All analyses were performed using statistical software SAS version 9.4 (SAS Institute, Cary, NC, USA) and GraphPad (GraphPad Software Inc., La Jolla, CA, USA).

Results

Demography

Comparative analyses of baseline demographic and clinical characteristics of the patients in the bleeder group (n=14) and non-bleeder group (n=33) are summarized in Table 1. The percentage of black patients was higher in the bleeder group. Body weight of the patients in the bleeder group was lower than those in the non-bleeder group. Thus the body mass index and body surface area were found to be significantly lower in the bleeder group compared to the non-bleeder group. Both the non-bleeder and bleeder groups were comparable with each other with respect to age, sex, history of smoking and substance abuse, vital signs, etiology of heart disease, echocardiographic parameters, PFA-100 and TEG tests.

Laboratory Hematology and Blood Chemistry

At the baseline (Pre-OP) there were no significant differences between groups in all hematological and blood chemistry parameters presented in figure 1. Post implant leukocyte counts were significantly increased temporarily at POD-3W and POD-1M in the bleeder group compared to the non-bleeder group ($p<0.05$). Erythrocyte, hemoglobin and hematocrit values were reduced temporally in both the non-bleeder and bleeder groups with similar trends. Platelet count found to be significantly decreased in bleeder group at POD-2W, 3W and 1M. We did not notice any temporally significant differences in INR, PTT between the two groups presented in Figure 1. This might suggest that the anticoagulation and antiplatelet treatment did not have a significant impact on bleeding complications.

Status of oxidative stress

The data for oxidative stress status in the healthy volunteers and HF patients are presented in table 2. The HF patients in the bleeder and non-bleeder groups had a preexisting condition of elevated oxidative stress compared to the healthy volunteers as evident from significantly higher ($p<0.05$) levels of ROS & oxLDL and concomitantly lower levels of SOD and TAC. However, these indicators for the oxidative stress were more prominent ($p<0.05$) temporally in the bleeder group at pre- and post-implant times compared to the non-bleeder group (Table 2).

Platelet integrin $\alpha 2b\beta 3$: surface expression, shedding and activation

Figure 2A shows the flow cytometric strategy to investigate expression of integrin $\alpha 2b\beta 3$ on the platelet surface. At the baseline, the surface expression of integrin $\alpha 2b\beta 3$ was significantly lowered in the bleeder group when compared to the non-bleeder group and healthy control, indicating a pre-existing condition of low $\alpha 2b\beta 3$ receptors in the bleeder group prior to CF-LVAD implantation (Fig. 2B). While looking at the post implant surface

expression, we noticed a progressive decrease in the surface expression of integrin $\alpha 2b\beta 3$ in the bleeder group and became lowest at POD-1M. But in case of the non-bleeder group, we did not notice any temporal change during the study time course of CF-LVAD support. The temporal change in integrin $\alpha 2b\beta 3$ on the platelet surface remained always significantly lowered in the bleeder group in comparison to the non-bleeder group (Fig. 2C). Contrary to the reduced surface expression of integrin $\alpha 2b\beta 3$, we noticed significantly elevated plasma levels of integrin $\alpha 2b\beta 3$ in the bleeder group compared to the non-bleeder group and healthy volunteers at baseline (Fig. 2D). Moreover, the post-implant temporal changes in plasma integrin $\alpha 2b\beta 3$ were found to be significantly higher at all the time points in the bleeder group compared to the non-bleeder group (Fig. 2E).

Figure 3A shows the flow cytometric analysis of integrin $\alpha 2b\beta 3$ activation on the platelet surface. At baseline, compared to the healthy volunteers, integrin $\alpha 2b\beta 3$ activation (MFI of PAC-1 positive platelets) in the non-bleeder and bleeder groups were not statistically significant. We noticed that integrin $\alpha 2b\beta 3$ activation was significantly higher in the bleeder group compared to the non-bleeder group prior to CF-LVAD implantation (Fig. 3B). After CF-LVAD implantation, integrin $\alpha 2b\beta 3$ activation temporarily increased at each week in case of the non-bleeder group. While looking at the bleeder group, it increased at POD-1W and then decreased temporarily. After one week, the activation pattern of integrin $\alpha 2b\beta 3$ became opposite to each group temporarily.

Association between oxidative stress and integrin $\alpha 2b\beta 3$

To investigate the relationship between the biomarkers of oxidative stress and platelet integrin $\alpha 2b\beta 3$ expression, shedding and activation, we conducted the Spearman's rank correlation test in all the CF-LVAD patients (Table 3). Our data clearly indicated that the surface expression of integrin $\alpha 2b\beta 3$ correlated negatively with generation of ROS and level of oxLDL; and correlated positively with concentration of SOD and TAC ($p < 0.0001$). As expected, the opposite strong correlations were evident in case of integrin $\alpha 2b\beta 3$ shedding and oxidative stress markers. The activation of integrin $\alpha 2b\beta 3$ correlated negatively with ROS, SOD and TAC; and correlated positively with oxLDL; the last two (TAC, oxLDL) were found to be statistically significant (Table 3). In multivariate regression analysis, ROS and oxLDL were significantly associated with platelet integrin $\alpha 2b\beta 3$ shedding after controlling for gender, race and BMI as possible confounders ($p < 0.001$).

Likelihood of MBC prediction model of integrin $\alpha 2b\beta 3$ shedding

The MBC model was developed post-hoc and was not part of the initial hypothesis. Based on our findings of association of integrin with risk bleeding we subsequently hypothesized that likelihood of bleeding depends on increase of integrin concentration from baseline and as well number of times it will increase. We developed an equation using the variables of change in concentration and frequency. We evaluated the marker by change in their value at these time points from baseline value. We hypothesized that scale of increase in the integrin $\alpha 2b\beta 3$ shedding from the baseline level as well as the frequency of increase from baseline is proportional to the likelihood of bleeding. First, we calculated the difference of concentration from baseline at each available follow-up time points i.e. POD-1W, 2W, 3W and 1M. Then we calculated the mean of the difference in those follow-up time point

concentrations. We also assessed the frequency of increase in the concentrations from baseline. We then used following equation to calculate likelihood of bleeding for integrin $\alpha 2b\beta 3$ shedding.

$$\text{Likelihood of MBC} = \frac{[(\text{mean } \Delta \text{conc.}) * (f \uparrow \text{conc.})]}{(f_n)}$$

Where,

conc. = mean difference in concentration from baseline

$f \uparrow_{\text{conc.}}$ = frequency of increased concentrations from baseline

f_n = total frequency of follow-up concentrations

Estimation of ROC curves for Likelihood of bleeding prediction model

We used the likelihood bleeding in a multivariate logistic regression model to evaluate its relation to the bleeding. The ROC curve for the integrin $\alpha 2b\beta 3$ likelihood of bleeding unit is shown in Figure 4. The estimated AUC was 0.8263 (95% CI: 1.011–1.117). According to this statistic, if we randomly select a patient with MBC and a patient without MBC, the probability of major bleeding (while controlling patient characteristics like age and BMI) being greater for the bleeding patient than that of non-bleeding patient is 0.8263. To test whether the AUC was significantly different from 0.5, a wald Chi-square statistic of 5.78 was obtained (using the estimated standard error of 0.0254), which yields a p value of 0.0162, significant at a Type I error rate of 5%. Thus the discriminating power of the biomarker of the predicted probability for bleeding by the integrin $\alpha 2b\beta 3$ likelihood of bleeding unit is significantly greater than that of chance alone. Thus, the measured integrin $\alpha 2b\beta 3$ shedding as a potential biomarker has a predictive power for MBC.

Discussion

CF-LVADs have improved patient outcomes and decreased post-operative complications compared to patients supported with older pulsatile devices. However, MBC has increased, which results in significant morbidity [36]. The incidence of MBC in CF-LVAD patients varies from 18% to 40% [36]. The potential causes of bleeding are likely multi-factorial. Prior studies showed that almost all HF patients with CF-LVAD support developed acquired von-Willebrand Syndrome due to a loss in high molecular weight vWF multimers, which may result in reduced vWF associated platelet activity and aggregation [37–39]. However, not all CF-LVAD patients experienced MBC. In the present study we examined oxidative stress status and deficiency in platelet surface expression or shedding of integrin $\alpha 2b\beta 3$ and their association with MBC among CF-LVAD patients.

Platelets are unique blood element without having a nucleus, but contain mitochondria, and play major role in hemostasis and thrombosis [40]. Platelet integrin $\alpha 2b\beta 3$ is the most abundant surface-expressed integrin (40,000–80,000 copies per platelet) [41]. The crucial importance of integrin $\alpha 2b\beta 3$ is clearly demonstrated by the studies of the genetic disease, Glanzmann thrombasthenia, in which platelets lack this integrin [42] and genetically

engineered mice lacking $\alpha 2b\beta 3$ [43] both have major bleeding problems and their platelets fail to aggregate. The balance between hemostasis and thrombosis relies on a finely tuned adhesive response of blood platelets. Inadequate adhesion leads to bleeding, whereas excessive or inappropriate adhesion leads to thrombosis [44]. We noticed temporarily reduced surface expression and elevated plasma levels of platelet integrin $\alpha 2b\beta 3$ in the patients experiencing MBC when compared to those without bleeding complications, - indicating possibilities of proteolytic shedding during the CF-LVAD support. Thus, the decreased number of integrin $\alpha 2b\beta 3$ on platelet surface may produce abnormal platelets which may facilitate the process of MBC in CF-LVAD patients.

With respect to the surface expression, activation and shedding of the platelet integrin $\alpha 2b\beta 3$, our study clearly showed that significantly higher activation of integrin $\alpha 2b\beta 3$ in the bleeder group compared to the non-bleeder up to one week of CF-LVAD support. After one week of CF-LVAD support, $\alpha 2b\beta 3$ activation gradually decreased temporally in the bleeder group but increased temporally in the non-bleeder group. While some of the integrin $\alpha 2b\beta 3$ could be shed from the surface, a portion of the remaining receptors might be activated at the same time. The activation of integrin $\alpha 2b\beta 3$ would enhance the platelet aggregation and adhesion, leading to thrombotic potential. On the other hand, the shedding of integrin $\alpha 2b\beta 3$ could reduce the platelet normal hemostatic function, leading to bleeding [45].

Redox control of cellular homeostasis is a key determinant of platelet destiny [46]. Platelet activity and fate have been reported to be strictly controlled by ROS formation and redox reactions [47–51]. The increased formation and release of intraplatelet ROS, as well as the modified content of endogenous or exogenous antioxidants may play important role not only in platelet destiny and thrombus formation, but also in the development of other diseases conditions. Significantly higher intraplatelet ROS generation and elevated plasma levels of oxLDL in the bleeder group compared to the non-bleeder group were observed in our study. We also noticed a concomitant decrease in the potent antioxidant enzyme SOD, and TAC; suggesting that the bodies antioxidant capacity might not be strong enough to minimize the deleterious effect of oxidative stress. Integrin $\alpha 2b\beta 3$ is one of the platelet receptors that are known to be regulated by redox systems [51]. In univariate analysis, strong correlations between the biomarkers of oxidative stress and platelet integrin $\alpha 2b\beta 3$ shedding in the HF patients were observed in our study. Moreover, in multivariate regression analysis, oxidative stress was significantly associated with platelet integrin $\alpha 2b\beta 3$ shedding after controlling possible confounders like gender, race and BMI. Thus, our study may suggest that oxidative stress may play a role (at least in part) in accelerating integrin $\alpha 2b\beta 3$ shedding leading to platelet dysfunction in CF-LVAD patients and subsequent MBC. We further verified the predictability of the likelihood of MBC using the platelet integrin $\alpha 2b\beta 3$ shedding values and performed ROC analysis. Based on this statistical analysis, the estimated AUC from the ROC curve of the likelihood of MBC is 0.8263 (95% CI: 1.011 to 1.117). According to the common statistical standard for the AUC, the likelihood of MBC derived from integrin $\alpha 2b\beta 3$ shedding values would be a good biomarker as a diagnostic test for MBC in CF-LVAD patients.

Platelet integrin $\alpha 2b\beta 3$ shedding could be due to other factors such as long-term exposure to the high shear stress flow environment as well as the artificial blood contacting materials

found in CF- LVADs. Recently, it was shown that the non-physiological high shear stress (NPHSS) environment that exists in CF-LVADs may be responsible for platelet dysfunction as evidenced by GPIIb/IIIa, GPVI and platelet integrin $\alpha 2\beta 3$ receptor shedding [17–19]. Understanding of the role of oxidative stress and NPHSS from CF-LVAD as contributory factor in platelet integrin $\alpha 2\beta 3$ shedding may enable development of effective medical management strategy.

We acknowledge that there are some limitations in this prospective observational study. The sample size was relatively small. Not all CF-LVAD patients were enrolled for this study. Medication might have some impact on oxidative stress status. Recent changes in medications should be addressed in further studies to rule out drug drug interactions or a new side effect. A larger cohort of CF-LVAD patients with bleeding complication is needed to confirm our initial findings.

Acknowledgments

The study was partially supported by the National Institutes of Health (Grant Number 1R01HL124170).

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Highlights

- Oxidative stress and integrin $\alpha 2b\beta 3$ shedding were observed in CF-LVAD patients
- Oxidative stress is positively associated with integrin $\alpha 2b\beta 3$ shedding
- Integrin $\alpha 2b\beta 3$ shedding has a predictive power of major bleeding complications
- Integrin $\alpha 2b\beta 3$ shedding may be responsible for major bleeding in CF-LVAD patients

Figure 1.

Temporal changes in key routine laboratory hematology and blood chemistry parameters at the baseline (Pre-OP) and multiple time points after CF-LVAD implantation in non-bleeder and bleeder groups. Data are expressed as mean $\pm$ SD. The dotted lines in each diagram indicating the reference range of each parameter measured.

Figure 2.

Change in platelet surface integrin $\alpha 2b\beta 3$ expression and shedding among healthy volunteers, non-bleeders and bleeders at baseline and multiple time frames after CF-LVAD implantation. (A) Platelet population were identified in whole blood according to their forward scatter/sideward scatter (FSC/SSC) properties and positive staining for CD41. Surface integrin $\alpha 2b\beta 3$ expression was evaluated from CD41 positive platelet population with specific integrin $\alpha 2b\beta 3$ antibody and the data are presented as MFI in *arbitrary units*. Shift of fluorescence intensity from healthy control to non-bleeder to bleeder indicating reduction of surface expression. (B,D) Box-whisker plots showing control and baseline values of platelet surface expression and plasma levels of integrin $\alpha 2b\beta 3$ in non-bleeder and bleeder groups. The lines across each box plot represent the median value. The lines that extend from the top and the bottom of each box represent the lowest and highest observations still inside the lower and upper limit of confidence. (C,E) Change in post-operative integrin $\alpha 2b\beta 3$ expression and shedding between non-bleeder and bleeder during the study period. Data are presented as median with *interquartile range* (IQR). $*p < 0.05$ is considered significant.

Figure 3.

Change in activation of surface integrin $\alpha 2\beta 3$ (PAC-1 activation) among healthy volunteers, non-bleeders and bleeders at baseline and multiple time frames after CF-LVAD implantation. (A) Platelet population were identified in whole blood according to their forward scatter/sideward scatter (FSC/SSC) properties and positive staining for CD41. Integrin $\alpha 2\beta 3$ activation was evaluated from CD41 positive platelet population with specific PAC-1 antibody and the data are presented as MFI in *arbitrary units*. (B) Box-whisker plots showing control and baseline values of integrin $\alpha 2\beta 3$ activation in non-bleeder and bleeder groups. (C) Change in post-operative integrin $\alpha 2\beta 3$ activation between non-bleeder and bleeder during the study period. Data are presented as median with *interquartile range* (IQR). * $p < 0.05$ is considered significant.

Figure 4.

Receiver operating characteristic (ROC) curve for measurement of the predictive power of integrin $\alpha_2\beta_3$ shedding on major bleeding in CF-LVAD patients. AUC (area under the curve) was calculated using a method designed for ROC analysis.

Table 1

Demographic and baseline clinical characteristics of patients prior to CF-LVAD implantation

Pre-implant HF patients (n = 47)			
Characteristics	Non-bleeder group (n = 33)	Bleeder group (n =14)	<i>p-value</i>
<i>Demography</i>			
Age in years			
Mean ± SD	61.8 ± 8.1	59.5 ± 12.6	0.46
Median (IQR)	61.5 (42 – 76)	63.5 (25 – 70)	0.99
Sex, n (% male)	32 (97.0 %)	11 (78.6 %)	0.07
Race			
Caucasian white, n (%)	25 (75.8 %)	6 (42.9%)	0.05
Black, n (%)	8 (24.2 %)	8 (57.1 %)	
Height in meter			
Mean ± SD	1.8 ± 0.1	1.7 ± 0.1	0.14
Median (IQR)	1.8 (1.6 – 1.9)	1.7 (1.6 – 1.9)	0.11
Weight in kilograms			
Mean ± SD	100.1 ± 18.6	81.3 ± 17.4	0.01*
Median (IQR)	99.8 (60 – 127.9)	80.3 (53.0 – 112.0)	0.01*
Body mass index (BMI) (kg/m ²)			
Mean ± SD	31.1 ± 4.6	26.6 ± 4.9	0.01*
Median (IQR)	31.2 (20.8 – 39.0)	26.1 (19.5 – 34.3)	0.01*
Body surface area (BSA) (m ²)			
Mean ± SD	2.2 ± 0.2	2.0 ± 0.3	0.01*
Median (IQR)	2.3 (1.7 – 2.5)	2.0 (1.6 – 2.4)	0.01*
History of smoking, n (%)	7 (21.2 %)	3 (21.4 %)	1.00
History of substance abuse			
Ethyl alcohol abuse, n (%)	5 (15.2 %)	2 (14.3 %)	1.00
Drug abuse, n (%)	5 (21.2 %)	3 (21.4 %)	0.68
<i>Vital signs</i>			
Systolic blood pressure (mmHg)			
Mean ± SD	102.9 ± 11.3	107.7 ± 11.9	0.30
Median (IQR)	106 (79 – 129)	109 (86 – 131)	0.11
Diastolic blood pressure (mmHg)			
Mean ± SD	60.6 ± 7.7	67.6 ± 13.5	0.08
Median (IQR)	63 (46 – 74)	64 (60 – 105)	0.21
<i>Etiology of heart disease</i>			
Ischemic cardiomyopathy, n (%)	15 (45.4 %)	5 (35.7 %)	0.75
Non-ischemic cardiomyopathy, n (%)	9 (27.3 %)	5 (35.7 %)	0.73
Idiopathic Cardiomyopathy, n (%)	9 (27.3 %)	4 (28.6 %)	1.00
<i>Echocardiographic parameters</i>			

Characteristics	Pre-implant HF patients (n = 47)		
	Non-bleeder group (n = 33)	Bleeder group (n = 14)	p-value
Left ventricular end diastolic diameter (mm)			
Mean $\pm$ SD	65.6 $\pm$ 8.5	67.4 $\pm$ 11.7	0.67
Median (IQR)	63 (53 – 88)	67.5 (52 – 88)	0.60
Left ventricular ejection fraction (%)			
Mean $\pm$ SD	13.9 $\pm$ 3.4	15.0 $\pm$ 5.6	0.48
Median (IQR)	15 (10 – 20)	12.5 (10 – 25)	0.75
PFA-100 test parameters (Mean $\pm$ SD)			
PFA-CADP-CT (sec.)	150.8 $\pm$ 80.8	187.5 $\pm$ 85.1	0.17
PFA-CEPI-CT (sec.)	217.8 $\pm$ 84.9	253.3 $\pm$ 15.9	0.13
TEG-parameters (Mean $\pm$ SD)			
TEG-clotting time R Time (min)	5.3 $\pm$ 1.2	4.9 $\pm$ 2.3	0.44
TEG-K Time (min)	1.5 $\pm$ 0.2	1.8 $\pm$ 1.1	0.13
TEG-Fibrinogen activity (angle) degree	70.4 $\pm$ 2.9	66.6 $\pm$ 11.9	0.09
TEG-Maximum Amplitude (mm)	63.5 $\pm$ 5.4	59.2 $\pm$ 10.7	0.07

Demographic and clinical parameters of non-bleeder versus bleeder groups of HF patients were statistically compared by Mann-Whitney 'U' test (for median values with IQR), Fisher's Exact Test (for results presented as percentages) and Student's t-test (for results presented as mean $\pm$ SD) as applicable, *p<0.05 were considered significant.

Table 2

Status of oxidative stress biomarkers between healthy volunteers and heart failure patients at baseline and multiple time frames after CF-LVAD implantation in both non-bleeder and bleeder groups.

Biomarkers of oxidative stress	Healthy volunteers (n=15)	HF patients (n=47): Non-bleeder (n=33) vs. Bleeder (n=14)				
		Blood sample time points				
		Pre-OP	POD-1W	POD-2W	POD-3W	POD-1M
ROS	18.9 (10.0 – 90.4)					
Non-bleeder		137.2 (100.2 – 230.0) ^a	149.7 (100.58 – 200.2)	115.9 (88.71 – 162.8)	137.1 (100.0 – 171.4)	115.9 (87.9 – 168.9)
Bleeder		183.9 (138.9 – 230.5) ^{a,b}	173.9 (111.8 – 255.8) ^b	200.1 (128.9 – 258.2) ^b	217.4 (128.7 – 333.8) ^b	261.4 (172.6 – 305.9) ^b
oxLDL	44.7 (30.8 – 51.7)					
Non-bleeder		89.3 (55.9 – 124.3) ^a	110.7 (98.2 – 131.4)	103.9 (70.8 – 131.2)	109.0 (68.4 – 141.7)	136.5 (119.8 – 151.2)
Bleeder		108.3 (88.3 – 124.1) ^{a,b}	138.2 (118.7 – 151.2) ^b	159.8 (144.2 – 174.5) ^b	176.1 (144.3 – 191.2) ^b	190.7 (169.8 – 201.4) ^b
SOD	387.4 (321.3 – 411.3)					
Non-bleeder		200.3 (168.7 – 288.7) ^a	188.9 (155.8 – 211.4)	200.2 (178.8 – 241.2)	210.7 (188.9 – 255.1)	199.6 (175.4 – 213.2)
Bleeder		185.8 (155.4 – 247.3) ^{a,b}	155.4 (144.3 – 181.1) ^b	134.2 (124.2 – 145.2) ^b	138.2 (131.2 – 155.2) ^b	111.6 (101.4 – 121.4) ^b
TAC	1.4 (1.2 – 1.8)					
Non-bleeder		1.3 (1.1 – 1.5) ^a	1.0 (0.6 – 1.1)	1.2 (1.1 – 1.41)	1.0 (0.7 – 1.3)	1.2 (0.9 – 1.4)

Biomarkers of oxidative stress	Healthy volunteers (n=15)	HF patients (n=47): Non-bleeder (n=33) vs. Bleeder (n=14) Blood sample time points				
		Pre-OP	POD-1W	POD-2W	POD-3W	POD-1M
Bleeder		1.1 (0.6 – 1.4) ^{a,b}	0.8 (0.5 – 1.1)	0.9 (0.6 – 1.1) ^b	0.6 (0.4 – 0.9) ^b	0.6 (0.4 – 0.9) ^b

Note: Results are expressed as median values with interquartile ranges in parentheses.

^{a,b} p<0.05 is considered significant.

^a healthy volunteers vs. Pre-OP;

^b non-bleeder vs. bleeder at each blood sample time point.

ROS: platelet reactive oxygen species in terms of mean fluorescence intensity (MFI) in arbitrary units; SOD: superoxide dismutase as U/mL; TAC: total antioxidant capacity as mmol/L; oxLDL: oxidized low-density lipoproteins as U/L.

Table 3

Spearman correlation between biomarkers of oxidative stress and platelet integrin $\alpha 2b\beta 3$ in CF-LVAD patients.

Biomarkers of oxidative stress	Platelet integrin $\alpha 2b\beta 3$		
	$\alpha 2b\beta 3$ expression	$\alpha 2b\beta 3$ shedding	$\alpha 2b\beta 3$ activation
ROS			
Spearman's ρ	0.54	0.31	0.09
95% confidence intervals (CI)	0.6528 to 0.4010	0.1193 to 0.4868	0.2617 to 0.08590
p-value	< 0.0001 *	0.0015 *	0.2993
SOD			
Spearman's ρ	0.39	0.37	0.12
95% confidence intervals (CI)	0.2029 to 0.5481	0.5418 to 0.1697	0.3086 to 0.08856
p-value	<0.0001 *	0.0004 *	0.2538
TAC			
Spearman's ρ	0.59	0.45	0.31
95% confidence intervals (CI)	0.4583 to 0.6921	0.5927 to 0.2819	0.4580 to 0.1385
p-value	<0.0001 *	<0.0001 *	0.0003 *
oxLDL			
Spearman's ρ	0.56	0.46	0.29
95% confidence intervals (CI)	0.6697 to 0.4247	0.2953 to 0.6021	0.1175 to 0.4410
p-value	<0.0001 *	<0.0001 *	0.0008 *

Note: Result was expressed as ρ (rho) value.

* statistically significant in spearman's rank correlation test.