Title: Hemolysis and Platelet Activation during Continuous Flow Mechanical Circulatory Support (CF MCS)

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[redacted]

Background:

Heart failure (HF) has reached endemic proportions effecting over 5.4 million people worldwide and its burden is projected to exponentially grow in the next decade [1]. HF is a progressive syndrome and despite improvements in medical therapy, most patients will develop advanced HF overtime. With implementation of the current generation of durable CF pumps, the 2-year survival for patients with chronic advanced HF has improved to nearly 70% [2]. For patients in acute cardiogenic shock, CF pumps such as veno-arterial extracorporeal membrane oxygenation (VA-ECMO) or Impella have become a major mode of temporary cardiac support until recovery, durable CF pump implantation or transplantation. Utilization of VA-ECMO has grown by over three-fold during the prior decade with survival outcomes of 40-50% for otherwise moribund patients; however, these outcomes have remained stagnant [3]. Despite these remarkable improvements in survival with durable CF pumps and the clear lifesaving effects of Impella and VA-ECMO, serious adverse hematological events such as bleeding and thrombosis create substantial morbidity and mortality and remain major barriers for further expansion of this technology. In particular, thrombosis is a devastating adverse event during CF pump support as it can lead to stroke, device stoppage, and hemodynamic collapse. Although the annual incidence of pump thrombosis has been reported to range from 8 to nearly 30% [4, 5] the pathobiological mechanisms of thrombus formation during CF pump support with ongoing anticoagulation remain elusive [4, 6].

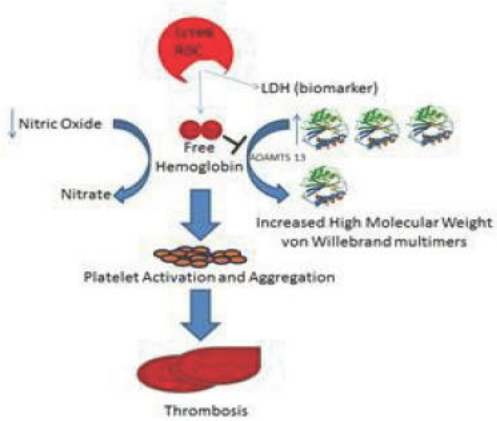

Figure 1: A. Pathway of free hemoglobin induced thrombus formation during diseases of intravascular hemolysis such as sickle cell anemia. Free hemoglobin released from red blood cell lysis depletes nitric oxide (NO) by converting it to nitrate. Free hemoglobin binds ultra large (UL) and high molecular weight (HMW) multimers of vWF to prevent their proteolysis by ADAMTS 13. Reduced NO and more UL multimers create a pro-thrombotic state by increasing platelet activation and aggregation leading to thrombus formation. LDH is released from red blood cell lysis and serves as a biomarker for hemolysis.

Hemolysis and thrombotic events:

The incidence of hemolytic events during durable CF pump support is estimated to be 15-35% and may affect as many as 1 in every 3-4 patients [7-9]. We have previously shown that a majority of pump thrombosis events are from non-mechanical etiologies and occur more frequently with pre-existing pro-thrombotic conditions. Several investigations show elevated LDH levels in patients many weeks before pump thrombosis is apparent clinically [4, 7-9]. After durable CF pump implantation, LDH levels are clinically screened biweekly and a rise to >700 U/L, prompts further exploration of mechanical and non-mechanical etiologies. Although conventional wisdom in the MCS field has been that hemolysis is a result of

thrombus obstructing blood flow, such observations have led to speculation that hemolysis mayprecede and indeed trigger thrombus formation.

Pathophysiology of hemolysis induced thrombus formation: It is conceivable that low-grade hemolysis occurring in CF pumps due to transient turbulence, high shear stress, increased afterload and/or heat related fibrin deposition could be the inciting events for thrombus formation.
Specifically, following the release of free hemoglobin with its potent inhibitory effects on nitric oxide (NO) bioavailability and stabilization of pro-thrombotic ultra large (UL) and high molecular weight (HMW) vWF multimers, an increase in platelet activation and aggregation may occur (**Figure 1**). The preceding sequence of events triggered by free hemoglobin has been well described in diseases of ongoing hemolysis such as sickle cell anemia (SCA) and paroxysmal nocturnal hemoglobinuria (PNH) [10, 11]. Furthermore, recent *in vitro* studies reveal that exposure of purified platelets to red blood cell hemolysate or extracellular free hemoglobin increases platelet activation and aggregation in a dose dependent manner. Slepian and colleagues (collaborators on this proposal) showed through platelet aggregometry that increasing concentrations of plasma free hemoglobin and RBC hemolysate increased PA/A (**Figure 2**) [12].

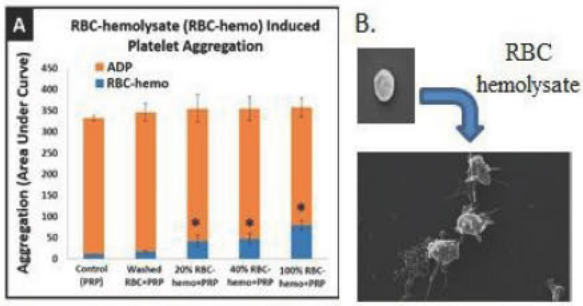

Figure 2: Platelet aggregation induced by RBC-hemolysate (blue bars) and maximal platelet aggregation mediated by additional adenosine diphosphate (orange bars). B. Scanning electron microscope of platelet aggregation after exposure to RBC hemolysate. (images courtesy of Dr. Slepian).

Inhibition of NO and increased platelet activation by extracellular free hemoglobin:

NO is produced by endothelial as well as circulating blood cells and its hematologic functions include inhibition of platelet activation, aggregation, and release of procoagulant proteins. Free hemoglobin directly limits NO bioavailability by oxidizing it to nitrate [13]. NO represses platelet activation and when its bioavailability is reduced during ongoing hemolysis, the propensity of platelet activation and aggregation is increased, creating a nidus for thrombus formation [10]. NO also activates guanylate cyclase producing cGMP and activating G kinase. G kinase in turn phosphorylates the thromboxane (TXA_2) receptor on platelet membranes which disrupts coupling of this receptor to its GTP binding protein G_q to inhibit activation of effector phospholipase C, thereby preventing Ca^{2+} mobilization and platelet activation [14]. Moreover, inhaled NO in patients with Acute Respiratory Distress Syndrome has been shown to inhibit platelet aggregation by reduced binding to fibrinogen [15, 16]. Consistent with this model, sildenafil, which potentiates NO signaling by inhibiting phosphodieterase-5 (PDE-5) to prevent breakdown of cGMP, *decreases* platelet activation in patients with SCA [10, 17]. Moreover, inhibition of PDE-5 with sildenafil is known to improve endothelial dysfunction [18] which may further reduce platelet activation and thrombotic events such as stroke.

Possible role of Ultra Large (UL) and High Molecular Weight (HMW) multimers of von Willebrand Factor (vWF)in creating a prothrombotic state:

VWF is a very large protein (>20,000 kDa) consisting of 250 kDa subunits that polymerize in the Weibel Palade bodies within endothelial cells. It is constitutively released and can be broken down in the circulation to smaller multimers. For hemostasis, UL multimers activate and aggregate platelets to initiate thrombus formation, while HMW multimers have a more

homeostatic role. Excessive UL and HMW multimers are associated with thrombosis in conditions such as thrombotic microangiopathies [18] and sepsis-induced disseminated intravascular coagulation [19]. Conversely, loss of vWF HMW multimers leads to a bleeding diathesis as evident in congenital and acquired vWF deficiency disorders [20].

The breakdown of UL and HMW multimers is controlled by ADAMTS 13. Under conditions of supra-physiologic shear stress, such as during CF pump support, the tensile force on VWF is increased by the square of the multimer length, thereby providing a mechanism for elongation and exposure of its cleavage site for ADAMTS 13 [21]. The resultant proteolysis of UL and HMW multimers leads to a type 2A VWF deficiency which is noted during CF pump support [22, 23]. Persistent hemolysis, which occurs during SCA and PNH, creates pro-thrombotic conditions by preventing breakdown of UL and HMW multimers [10, 11]. During hemolysis, free hemoglobin and its metabolic product, unconjugated bilirubin, can inhibit the normal cleavage of UL and HMW multimers by binding to them and preventing proteolysis by ADAMTS 13 [11, 24]. In patients with SCA, up to 14% of the total plasma VWF (4 fold higher than normal) is bound to extracellular hemoglobin [10, 11]. Additionally, hemoglobin-bound multimers are more adhesive to platelets and more hemostatic when assessed ex-vivo by the ristocetin co-factor and collagen binding assays [11] Furthermore, recent investigations with in-vitro high shear stress models that emulate CF pumps show that free hemoglobin increases VWF-mediated platelet adhesion [25]. Taken together, extracellular free hemoglobin released during hemolysis can bind to UL and HMW multimers to create a substantially pro-thrombotic substrate. Whether the observed hemolysis induced pro- thrombotic milieu during CF pump is related to reduced breakdown of UL and HWM multimers remains underexplored.

Research Protocol and Study Procedures

Aim 1: To evaluate LLH-related PA/A during CF MCS.

Design: In a cross-sectional format, we will recruit patients on chronic and acute CF MCS devices. Device- specific comparisons will be made to assess PA/A between those with and without LLH. In secondary analyses, we will also examine association of LLH with potential mediators of PA/A including activated GP IIb/IIIa receptor, p-selectin, platelet cGMP, vWF activity, vWF UL/HMW multimers and ADAMTS13 activity.

Population: Patients ≥18 years old on durable (HM2 and HM3) and acute (VA ECMO and Impella) CF MCS devices will be approached for participation. We will only approach HM3 patients that are commercially implanted and not enrolled in an ongoing clinical trial. Patients receiving sildenafil or nitrates for clinical indications will be excluded. Plasma Free Hemoglobin (PFHb) and Lactate Dehydrogenase (LDH) levels used to categorize patients as LLH (PFHb 10-20 mg/dL and LDH 400-700 U/L on three consecutive clinical measurements within a 14-day period to assure steady state) or no LLH (PFHb <10 mg/dL and LDH <400 U/L) are clinically measured daily in inpatients and every 2 weeks in outpatients at our institution.

Adult patients requiring acute CF pump support with veno-arterial (VA) ECMO or the Impella device for cardiogenic shock with an anticipated support time of at least 48 hours will be approached for participation in this study. The capacity to give informed consent will be evaluated by the study team. Patient's capacity will be assessed through the MacArthur

Competence Assessment.

Protocol: After acquiring informed consent from the patient, 8 am fasting blood (7 tubes, 3 ml per tube = 21 ml total, **table 1**) will be collected to measure PA/A. Newly implanted patients with durable devices will have blood samples drawn on the day of discharge and outpatients will be asked to come into the heart failure clinic for a scheduled research visit. In-patients with acute CF pumps will be screened and enrolled 48 hours after device implantation. For enrolled in-patients with ongoing LLH, we will also draw serial blood tests (3 tubes, 3 ml per tube = 9mL) daily for up to three days to monitor PA/A studies, to assess ongoing effects of LLH on platelets.

Table 1: Hematologic studies for Aim 1	Durable CF Pump HM2 (n=50) and HM3 (n=75)		Acute CF Pump VA ECMO (n=45) and Impella (n=45)	
	LLH (HM2 n=15; HM3 n=15)	no LLH (HM2 n=35; HM3 n=60)	LLH (n=15 per device)	no LLH (n=30 per device)
PA/A studies (3 light blue top tubes) -Aggregometry -Flow Cytometry: P Selectin and GP 2b3a in platelet rich plasma	x	x	x	x
Platelet cGMP measurement (1 blue top tube)	x	x	x	x
von Willebrand Factor electrophoresis and antigen/activity (2 light blue top tubes)	x	x	x	x
ADAMTS-13 activity (1 light blue top tubes)	x	x	x	x

Study Procedures: 1. PA/A studies: PA/A will be primarily measured by ex-vivo aggregometry (3 ml). In addition, secondary exploratory measures of PA/A will be assessed through flow cytometric detection of p-selectin and activated surface glycoprotein (GP) IIb/IIIA receptor. For platelet aggregometry and flow cytometry, studies will be promptly performed after specimen collection. Samples will be gently centrifuged to separate platelet-rich plasma (PRP) from white and red blood cells and platelet-poor plasma (PPP). PA/A will be assessed by a Multiple Analyzer (MA) after addition of an adenosine diphosphate (ADP) agonist to PRP. Although patients will be on aspirin, primary assessment of PA/A by aggregometry with ADP [>1µm] in aspirin exposed platelets is preserved [26]. Aggregation is plotted against time. The maximum height of the curve represents the aggregation level, the slope is the reaction velocity, and the area under the curve (AUC) is calculated as the main aggregation measure, given in arbitrary units (AU) [12]. This ADP induced AUC will be the primary measure of PA/A in this proposal. P-selectin is a cell-adhesion glycoprotein which is present in the alpha granules of platelets.

When platelets are activated, p-selectin localizes to the surface to mediate adhesion to other activated platelets, endothelial cells and leukocytes. Platelet membrane GPIIb/IIIa is a member of the integrin family of cell-membrane receptors that plays a key role in platelet aggregation and thrombus formation. Expression of activated GPIIb/IIIa receptors is exquisitely related to the PA/A. Both p-selectin and activated GP IIb/IIIa receptor in PRP will be measured through flow cytometry as previously described [17]. Prior to sample collection, 3 ml of blood will be wasted to eliminate the impact of venipuncture on PA/A. As the contemplated studies progress, the Platelet Activity State (PAS) Assay, which has been defined by our collaborating group by Dr. Slepian, will be added as an exploratory measure. The PAS assay evaluates mechanical shear-mediated platelet activation [27, 28].

2. Platelet cGMP: Whole-blood samples (3 ml) anticoagulated with citrate will be centrifuged to obtain PRP. Platelet cGMP will be quantified as described previously [35] by cGMP ELISA Kit (Cayman Chem., USA).

3. VWF studies: Standard gel electrophoresis will be conducted on plasma samples (3 ml). A

polyclonal rabbit antihuman antibody to vWF from Dako Inc. will be used to identify vWF multimers' bands which will be further quantified by densitometry. VWF activity will be measured from plasma (3ml) as the ratio of vWF collagen binding activity (measures binding of vWF to collagen as a function of the quantity of HMW multimers) to vWF antigen.

4. ADAMTS13 testing: Whole-blood samples (3 ml) will be centrifuged, and plasma will be removed. After repeat plasma centrifugation to remove platelet contamination, plasma specimens will be assessed for ADAMTS13 activity. This will be measured by a fluorescence resonance energy transfer (FRET)-based assay using a 73 amino-acid peptide (FRETS-VWF73) of von Willebrand factor (vWF) as a substrate [29].

**Plasma specimens for platelet cGMP, vWF and ADAMTS 13 activity studies will be frozen at -80°C and stored for analysis every week in batches during study period.*

5. LDH and plasma free hemoglobin are clinically measured daily on all inpatients and every 2 weeks in all outpatients with CF pumps by our institution's coagulation laboratory. As stated in the career and training plan, the candidate will be trained in the Yazdanbakhsh, Slepian, and Reyes labs to become adept in platelet aggregometry, flow cytometry, the PAS assay and vWF and ADAMTS 13 activity studies. Then the proposed studies will be performed by the candidate at the home institution in Dr. Reyes laboratory. In addition, a laboratory technician will be employed and trained to further assist with platelet aggregometry, flow cytometry (p-selection, GPIIb/IIIa receptor), platelet cGMP ELISA assays, VWF and ADAMTS13 activity studies.

Statistical analysis, sample size calculations and timeline: The primary measure for PA/A is ADP-induced AUC assessed by platelet aggregometry. ADP-induced AUC will be compared between the LLH and no LLH groups with an unpaired Student's t test. The null hypothesis is that there will be no difference in the PA/A between groups. A prior in-vitro study suggested that LLH increases PA/A by at least 30% as measured by aggregometry from 20±5 to 26±4 AU [12]. If this is the true population effect of LLH on PA/A in patients with CF pumps, with aminimum of 15 patients in each group per device we would achieve **90% power** to detect asignificant difference in means with a two-sided alpha of 0.05. Since there will be fewer patients withLLH, we will recruit for both groups until we have enrolled 15 patients in the LLH group for each device. As device specific comparisons will be made, for durable CF pumps we estimate that we will have to screen 80 HM2 and 115 HM3 patients. We estimate that 35% of these patients will be excluded due to being on sildenafil, nitrates, active infection or withholding consent. This will leave 50 HM2 and 75 HM3 patients available for analysis. From historical data [30], we expect that approximately 30% of HM2 (n=15) patients and 20% of HM3 (n=15) will have LLH and the remainder will be in the no LLH group (35 HM2, 60 HM3). For acute CF pump support, we estimate that 90 VA ECMO and 90 Impella patients will need to be screened and after 50% exclusion, 45 VA ECMO and 45 Impella will be enrolled and available for analysis. Based on our preliminary data (**Figure 3C**) for VA ECMO and prior reports in Impella [31], we expect that at least 35% of patients for each acute CF pump (n=15 per device) will have LLH and the remainder will be in the no LLH group (n=30). At our institution there are 5 durable CF pumps implants (3 HM3 and HM2) and 6 acute support CF pumps (3 VA ECMO and 3 Impella) implanted every month. In addition, there are currently 125 stable outpatients with durable CF pumps (95 HM2 and 30 HM3) available for screening. Therefore, we estimate it would take 32 months to enroll and analyze the calculated number of patients for all devices (see timeline). For each device specific comparison, we will examine the confounding effects of age, sex, body mass index, race/ethnicity (such variables are known to effect

thrombosis during CF MCS) by comparing them between LLH and no LLH groups. If significant differences are found then adjusted multivariable models will be used to assess the independent association of LLH and PA/A. We will also assess if there are device differences by conducting a 1-way ANOVA test of the difference PA/A between LLH and no LLH amongst all 4 device groups and post-hoc comparisons if any significant difference is observed. Results from devices that do not show heterogeneity will be pooled to assess the association across device type. Similar to the device specific analysis noted above, confounders will also be examined, and if appropriate, adjusted for in this pooled sample. Lastly, we will compare for each device and in any pooled analysis, the association of LLH with potential mediators of PA/A including p-selectin, activated GP IIb/IIIa receptor, cGMP, vWF activity, UL/HMW multimers, ADAMTS 13 activity by unpaired Student's t tests.

Anticipated results and experimental problems: We expect that patients with LLH will have a significant increase in PA/A measured by ADP-induced AUC and associated higher p-selectin, activated IIb/IIIa receptor, lower levels of platelet cGMP, higher vWF activity, greater presence of UL/ HMW multimers, and lower ADAMTS 13 activity in comparison to those with no LLH. If an increase in PA/A is not observed at the selected LDH and PFHb thresholds, we may reanalyze the exposure impact of LLH as a continuous variable. If ADP induced PA/A is not observed then we may try other agonists to induce PA/A including TRAP, epinephrine, collagen, and arachidonic acid. As an alternative strategy, exogenous platelet activation on PRP will be assessed with PPP to evaluate for other hemolysate products that may impact PA/A. If cGMP is unchanged, then we will directly measuring NO bioavailability by Electron Paramagnetic Resonance (EPR) through FeDETC spin trapping methodology, which only captures the NO radical. If neither preceding platelet properties are changed then we will assess platelet agglutination, which is driven by vWF in a non-NO dependent mechanism. If changes in plasma UL/HMW multimers are not observed, we will further assess the ratio of HMW multimers to lower MW multimers. In addition, platelet-derived vWF which is also implicated in platelet activation and coagulation will also be analyzed. Lastly, with regard to sample size, if our historical projections on numbers of HM2 and HM3, or VA ECMO and Impella fall short, we can increase recruitment an additional 6-12 months to achieve target enrollment.

Aim 2: To determine the impact of targeted anti-platelet therapy with sildenafil on changes in PA/A and endothelial function during CF MCS mediated LLH.

Design: Prospective, randomized (1:1), double-blind study investigating the effect of a 15-day treatment with (sildenafil) or matched placebo on PA/A in patients with ongoing LLH on durable CF pump (HM2 and HM3).

Study Population - Inclusion criteria: Low Level Hemolysis (LLH) is defined as: abnormally elevated LDH $\geq$ 240 U/L, plasma free hemoglobin $\geq$10 g/dL, haptoglobin $\leq$ 100 mg/dL. Outpatients $\geq$18 years old with ongoing durable CF pump support (HM2 or HM3), off nitrates and sildenafil, will be eligible to participate in this study. If the radial pulse is palpable manually (pulsatile), then those with a systolic blood pressure (BP) <100 mmHg, taken by a manual blood pressure cuff, will be excluded. If the radial artery pulse is not palpable (non-pulsatile), then those with a Doppler blood pressure < 80 mmHg will be excluded. In non-pulsatile patients, the BP is taken by a Doppler ultrasound at the brachial artery and is closely correlated to the mean arterial BP. Those patients already on sildenafil or nitrates for clinical indications will also be excluded. In addition, patients with an ongoing infection that may predispose to thrombosis will be excluded as will those unwilling or unable to give written, informed consent.

Study Protocol: After obtaining informed consent, baseline demographics and hematologic biomarkers including fasting am PA/A and NO bioavailability measurements (3 tubes [1 tube for platelet aggregometry, 1 tube for exploratory biomarkers including p selectin, CD 40L, GPIIb/IIIa receptor, and 1 tube for NO bioavailability] x 3ml = 9 ml of blood) will be collected. In addition, endothelial function will be assessed by flow mediated dilation of the brachial artery and fingertip temperature changes following a 5 min blood pressure cuff inflation. Patients will be placed, using computerized randomization, into 2 groups to receive the study drug which will be either sildenafil or placebo. More precisely, an enrolled patient will be randomly placed in either the placebo or the sildenafil arm, both of which will be marked as "Study Drug." Neither the patient nor the drug administrator will be aware to which study arm the patient has been designated. Following randomization and if BP is in the acceptable range (see figure 4), 20 mg of the study drug will be administered. Then BP will be recorded every 30 mins for two hours. If BP drop is < 5 mmHg after 2 hours and patient is asymptomatic, they will proceed to take 20 mg of the study drug every 8, starting on day 2 (total 18 doses). Patients will be asked to return to the clinic on day 8 and 20 mg of the study drug will be administered. After 2 hours, which is the time of peak drug concentration, fasting am PA/A and NO bioavailability measurements (3 tubes [1 tube for platelet aggregometry, 1 tube for exploratory biomarkers including p selectin, CD 40L, GPIIb/IIIa receptor, and 1 tube for NO bioavailability] x 3ml = 9 ml of blood) will be collected. In addition, endothelial function will be assessed by flow mediated dilation of the brachial artery and fingertip temperature changes following a 5 min blood pressure cuff inflation. Then the patient will be asked to come back to clinic on the next weekday and if BP is in the acceptable range (see figure 4), 40 mg of the study drug will be administered. If BP remains stable for 2 hours (< 5 mmHg drop and asymptomatic), then patients will continue taking 40 mg every 8 hoursPatients will be asked to return to clinic on day 15. A final 40 mg dose of the study drug will be given and after 2 hours fasting am PA/A and NO bioavailability measurements (3 tubes [1 tube for platelet aggregometry, 1 tube for exploratory biomarkers including p selectin, CD 40L, GPIIb/IIIa receptor, and 1 tube for NO bioavailability] x 3ml = 9 ml of blood) will be collected. In addition, endothelial function will be assessed by flow mediated dilation of the brachial artery and fingertip temperature changes following a 5 min blood pressure cuff inflation. All patients will be on a standard antithrombotic regimen of aspirin 81 mg daily with a target INR of 2-2.5 IU.

Endothelial Function Testing will be conducted by non-invasive methodology. As has been previously well described [32], a vascular ultrasound probe will be used to assess the diameter of the brachial artery at the level of the antecubital fossa in the longitudinal view. Then an arm blood pressure cuff will be inflated beyond the systolic pressure for 5 minutes to cause transient ischemia. After deflation of the arm blood pressure cuff, the brachial artery diameter will be recorded again by the ultrasound vascular probe. In addition, by using the noninvasive Endothelix device, we will simultaneously also record figure tip temperature change before and after cuff deflation, as another measure of endothelial function.

Addendum to study protocol:

Aim 2 of this protocol is a randomized, blinded, placebo-controlled study to assess the effect of sildenafil on platelet function in patients supported by left ventricular assist devices. We originally proposed to enroll 46 patients into this study. At this point, 20 participants have successfully completed the study since enrollment which was initiated in 2019. Due to slower than expected enrollment for a variety of reasons, including the COVID-19 pandemic, a competing trial and a reduction in institutional implantation volume of these devices, it is no longer feasible to reach the originally proposed sample size within the planned study period

ending December 30, 2023.

In discussion with my mentoring team, we propose to amend the study protocol to perform an interim analysis at the current sample size of 20 participants. The statistical plan will be carried out as originally proposed in the study plan. If there is a trend at a $p<0.2$ for a change in platelet function between groups, then we will readjust the required sample size accordingly for the duration of the enrollment period. If this trend is not apparent, then it would be futile to enroll additional patients into this study aim and further enrollment will stop. In this scenario, we will proceed with analysis of proposed study measures with subsequent reporting of findings.

Statistical analysis, sample size calculations: The primary measure of PA/A is the change in ADP-induced AUC assessed by platelet aggregometry, which will be compared between the treatment and placebo groups by an independent samples Student's t test. The null hypothesis is that the mean change in PA/A will be the same in both groups at the 15 day time-point. In a prior single-arm study in patients with ongoing hemolysis from SCA, sildenafil reduced PA/A by around 40% from 30.6%±15.2 to 17.3%±12.4 [17]. If this is the true population effect with standard deviation of the sildenafil induced change in PA/A among patients on durable CF pump support and if there is no expected change in the placebo group, we would need 23 patients per group (46 total patients) to achieve **85% power** to detect a significant difference in mean reduction with a two-sided alpha of 0.05. Analysis will be by intention-to-treat. Although the primary analysis will be unadjusted, secondary analyses will apply linear regression models to adjust for age, sex, race/ethnicity, duration of device implant, type of device (HM2 or 3) and adherence. Given the small sample, adjustment will be limited to no more than 2 additional variables at a time. If device differences are observed, sub-group analyses by device will be performed. For secondary outcomes, the above analyses will be repeated at 8 days and also comparisons of changes in p-selectin, activated GP IIb/IIIa receptor and platelet cGMP at 8 and 15 days.

Plan for recruitment, retention, timeline and check points: Patients on CF MCS are tracked closely in a centralized electronic medical registry kept by the HF group, which we will continue to leverage as a resource to identify patients. Currently, 52 outpatients identified through our CF MCS patient registry on durable devices with LLH are available for screening and at the current implant rate of 5 durable devices per month, 1 additional patient with LLH becomes available every month. With an estimated 35% ineligibility or withholding consent, 95 patients with LLH will need to be screened to enroll 62 patients. With an additional 25% drop-out rate after enrollment, this will leave 46 patients (23 per group) who will complete the study protocol. Since 52 outpatients with CF pumps and LLH are currently available (which is steady state for our program based on prior 3 years) and taking into account 35% ineligibility or unwillingness to participate at screening, we estimate that 34 patients can be immediately enrolled to get a head start. At the current availability of 1 new LLH patients per month, it will take an additional 42 months to screen and enroll the remaining patients. We will conduct checkpoints every 6 months examining if the anticipated enrollment (table 2) is being achieved. If enrollment is not met by 2 consecutive check points, we may increase recruitment an additional 6-12 months.

Anticipated results and experimental problems: We expect that enhancement of NO signaling with Sildenafil in patients with low level hemolysis will lead to a reduction in PA/A. If changes in PA/A are not observed, then exogenous PA/A will be assessed with platelet poor plasma. If PA/A is not affected, then follow-up experiments will have to be designed to identify the LDH and plasma free hemoglobin threshold value at which lower-level hemolysis increases PA/A.

Alternatively, if the expected reduction in PA/A is not observed with sildenafil we may try other pharmacological agents that also enhance NO signaling such as nitrates.

Risks:

The study involves initiating therapy with sildenafil which is already utilized during durable CF pump support in select patients with pulmonary hypertension without any significant known adverse events. A package insert for sildenafil is included in the IRB application. There are the risks associated with phlebotomy which include bruising and discomfort. To ensure confidentiality, all research data will be kept separate from the medical record in locked files with limited access. The 5 min arm ischemia blood pressure cuff method has been well described to assess endothelial function and is not known to be associated with adverse events [32]. In addition, the Endothelix has been cleared by the FDA.

Benefits:

Currently, limited data are available to explain the *in-vivo* mechanisms contributing to thrombosis during CF pump support. In this investigation we hypothesize that free hemoglobin released through hemolysis depletes NO, limits breakdown of pro-thrombotic UL multimers of vWF, and augments platelet function to create a nidus for thrombus formation. The preceding pathway is described in other diseases of ongoing hemolysis and *in-vitro* CF pump studies have also shown greater UL multimers and augmented platelet function triggered by free hemoglobin [10, 11, 25]. Confirmatory *in-vivo* evidence can lead to exploration of several therapeutic agents that may reduce thrombosis. For example, inhaled NO can be given to patients during ECMO support, oral agents that enhance NO signaling such as sildenafil or nitrates can be given to patients with hemolysis on durable CF pump support, and free hemoglobin scavenging agents such as haptoglobin and hemopexin can be infused during ongoing hemolysis [33]. Moreover, as opposed to biological diseases of ongoing hemolysis, the hemolysis stimulus can be removed during CF pump support through device exchange or explantation. In addition to unveiling free hemoglobin induced mechanisms of thrombosis, this proposal will investigate if enhancement of NO signaling can lead to reduced platelet activation and aggregation. In clinical practice we have noted a higher subsequent incidence of pump thrombosis and ischemic stroke in patients with ongoing low-level hemolysis (LDH > 384 U/L) after CF pump implantation which is mitigated inpatients receiving sildenafil for other clinical indications. Further CF LVAD experience with theHeart Mate 3, has also shown that low level hemolysis extends to any abnormal LDH level or can also be evident with haptoglobin level ≤ 100 mg/dL. If our hypothesis is confirmed, such patients may benefit from primary thrombosis prevention with augmentation of NO signaling by sildenafil or similar agents, which will be a novel approach to reduce thrombotic events during durable CF pump support.

Data Safety Monitoring Plan:

A data safety monitoring board (DSMB) will be created to review adherence to study protocol and breaks in confidentiality. In addition, any side effects or complications associated with phlebotomy such as bruising or with the initiation of sildenafil therapy including headache, dizziness and hypotension will be carefully tracked. Since sildenafil is typically well tolerated in patients with CF pumps the potential risks are likely minimal. The DSMB will be external and will consist of a heart failure attending cardiologist. [redacted].

Adverse event monitoring will be continuously done throughout the study period and a log of any AE's will be kept in an excel file. The DSMB will produce quarterly reports of adverse events that may have occurred during phlebotomy or after administration of drug therapy. To ensure accuracy of data, all adverse events will be verified [redacted]. If

greater than or equal to 2 patients experience any significant side effects of phlebotomy or sildenafil therapy, then the protocol will be halted until further notice by the DSMB. These adverse events will be communicated to the IRB by the PI (Saeed).

Alternative Therapies:

Patients may refuse participation in the study.

Compensation:

None. If requested, patients may be reimbursed for transportation.

Minors as Research:

It will be ensured that all patients will have been 18 years or older at the time of their enrollment.

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