von Willebrand Factor and Angiopoietin-2 are Sensitive Biomarkers of Pulsatility in Continuous-Flow Ventricular Assist Device Patients

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Nonsurgical bleeding occurs in a significant proportion of patients implanted with continuous-flow ventricular assist devices (CF-VADs) and is associated with nonphysiologic flow with diminished pulsatility. An *in vitro* vascular pulse pressure model seeded with adult human aortic endothelial cells (HAECs) was used to identify biomarkers sensitive to changes in pulsatility. Diminished pulsatility resulted in an ~45% decrease in von Willebrand factor (vWF) levels from 9.80 to 5.32 ng/ml (n = 5, $p < 0.05$) and a threefold increase in angiopoietin-2 (ANGPT-2) levels from 775.29 to 2471.93 pg/ml (n = 5, $p < 0.05$) in cultured HAECs. These changes are in agreement with evaluation of patient blood samples obtained pre-CF-VAD implant and 30-day postimplant: a decrease in plasma vWF level by 50% from ~45.59 to ~22.49 μg/ml (n = 15, $p < 0.01$) and a 64% increase in plasma ANGPT-2 level from 7,073 to 11,615 pg/ml (n = 8, $p < 0.05$). This study identified vWF and ANGPT-2 as highly sensitive to changes in pulsatility, in addition to interleukin-6 (IL-6), IL-8, and tumor necrosis-α (TNF-α). These biomarkers may help determine the optimal level of pulsatility and help identify patients at high risk of nonsurgical bleeding. *ASAIO Journal* XXX; XX;00–00

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Left ventricular assist devices (LVADs) have become a valuable therapeutic option for patients with advanced heart failure (HF), both as a bridge-to-transplantation and as destination therapy, due to the scarcity of donor organs.[1] Both types of LVADs, pulsatile-flow ventricular assist devices (PF-VADs) and continuous-flow VADs (CF-VADs), are similar in terms of providing hemodynamic support and improving the quality of life and functional capacity of patients.[2,3] Currently, almost all implanted VADs are continuous-flow pumps because they have critical advantages over older generation PF-VADs in terms of patient survival rate, higher durability, and smaller device and driveline size leading to lower rates of infection.[3] It has been estimated that LVADs could potentially be used to treat approximately 60,000 HF patients a year globally to improve their survival and enhance their quality of life. However, LVADs are greatly underutilized, with about 3,000 LVADs implanted in the US annually due to the risk of adverse events associated with LVAD treatment.[4] Specifically, CF-VADs create nonphysiological stimuli where the diminished flow pulsatility can lead to adverse arterial endothelial cell function. For example, reduced pressure mechanotransduction of the endothelial cells has been associated with several risks. Such as the increased possibility of hemolysis and pump thrombosis due to supraphysiological shear-induced platelet activation and nonsurgical bleeding, particularly in the gastrointestinal tract.[5] While major challenges still exist, hemolysis and pump thrombosis have been mitigated through alternative LVAD designs with reduced pump shear stress and residence times, repositioning the inflow cannula, and anticoagulant therapies.[6] Nonsurgical bleeding remains a frequent complication in patients supported with CF-VADs (18–40% of patients),[7,8] requiring transfusion of blood products (2–4 units)[9] and hospitalization.[10]

Currently, there is an incomplete understanding of the underlying mechanisms for nonsurgical bleeding in CF-VAD patients. However, clinical data suggest that the development of acquired von Willebrand factor syndrome (AVWS) and the formation of arteriovenous malformations (AVMs) are major risk factors.[11–15] One aspect that is likely underreported is the influence of AVWS on increased stroke risk in LVAD patients.[16] Many physicians typically reduce the anticoagulation and antiplatelet therapies in these patients to alleviate the repeated

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P.S., G.A.G., X.C., and K.T.N. developed the study conception and design. K.T.N., J.H., and I.C. contributed to the fabrication of microfluidic devices, and VPPM setup/operation. K.T.N., J.H., and L.D. contributed to the collection and processes of patient blood samples. K.T.N., J.H., and R.K. contributed to static culture of patient-derived cells. K.T.N. managed overall project, analyzed data, furnished figures, and prepared draft manuscripts. All authors reviewed the study results and approved the final version of the manuscript.

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nonsurgical bleeding that occurs due to endothelial von Willebrand factor (vWF) dysregulation.[16] Thus, AVWS-induced adverse events lead to decreased quality of life and enthusiasm for life-extending technologies like CF-VAD. Therefore, it is important to identify and characterize sensitive pulsatility biomarkers like vWF and proinflammatory/proangiogenic soluble factors. A greater understanding of these sensitive biomarkers may help rapidly detect and monitor patients at high risk of nonsurgical bleeding following CF-VAD placement. They can also help determine the optimal parameters for CF-VAD flow modulation protocols or other therapies that can mitigate nonsurgical bleeding events.

Small animal models do not faithfully replicate human physiology, and studies with large animal models like young calves and pigs do not result in nonsurgical bleeding. These models also do not represent the aging HF population that typically receives LVADs. Therefore, identifying and validating biomarkers sensitive to pulsatility require a model capable of replicating normal physiology and HF pathophysiology. In this study, we adopted a human vessel-on-a-chip model called the vascular pulse pressure model (VPPM), which enables the culture of human endothelial cells under conditions that simulate vascular pressure and flow pulsatility of patients with and without CF-VAD support. We used the VPPM to identify biomarkers that respond to physiologic and diminished pulsatility. Furthermore, we validated VPPM-identified biomarkers of pulsatility against biomarkers identified in blood samples collected from CF-VAD patients, pre- and post-CF-VAD implantations to assess biomarkers that show similar behavior both *in vitro* and in patients.

Materials and Methods

Microfabrication and Setup of Vascular Pulse Pressure Model

The VPPM is a unique closed-loop model developed in our lab based on principles of existing mock flow loops designed to mimic a human circulatory system and adapted for the culture of human endothelial cells. Previously, the VPPM was validated to recreate physiologic and selected flows associated with cardiovascular conditions for disease modeling and drug testing.[17,18] This study adopted the VPPM to culture human endothelial cells and identified biomarkers sensitive to pulsatility.

The VPPM microfluidic device (Figure 1A and B) utilizes a biocompatible polydimethylsiloxane (PDMS) material to function as a cell culture channel. The architecture of this device was designed using SolidWorks, then 3D printed into molds using a high-heat tolerance material, ceramic-like advanced high temp white (PerFORM), to withstand temperatures in the PDMS fabrication protocol. The printing process had high resolution using stereolithography technique and an industrial 3D printer at Protolabs, Inc. As a result, both molds resulted in smooth surfaces for device molding. The first mold builds the device's top piece with a 300 µm tall and 5 mm wide serpentine microfluidic channel. A second mold produces an approximately 1 mm thin bottom layer (Figure 1B). The purpose of this thin and flexible (~500 kPa) PDMS membrane is to replicate the distensibility and compliance of arterial vessels. Using an approximation for long rectangular membranes clamped on two sides,[19] we estimate that the maximum deflection at the center of membrane will range from 0.8 to 0.9 mm at the target pressures of 80–120 mmHg. These estimates are consistent with our previous measurements on similar devices.[17] For PDMS fabrication, the process involves thoroughly mixing PDMS monomers and curing agent (Product #: Qsil 216 A and Qsil 216 B, CHT USA Inc, USA) at a ratio of 10:1, which were then filled into both molds, degassed under vacuum for 60 mins, cured at room temperature overnight, and then heated to 70°C for 30 mins for complete polymerization of PDMS. The top PDMS piece was removed from its mold, and then two access holes were punched using syringe needles to create an inlet and outlet. Without removing the bottom membrane from

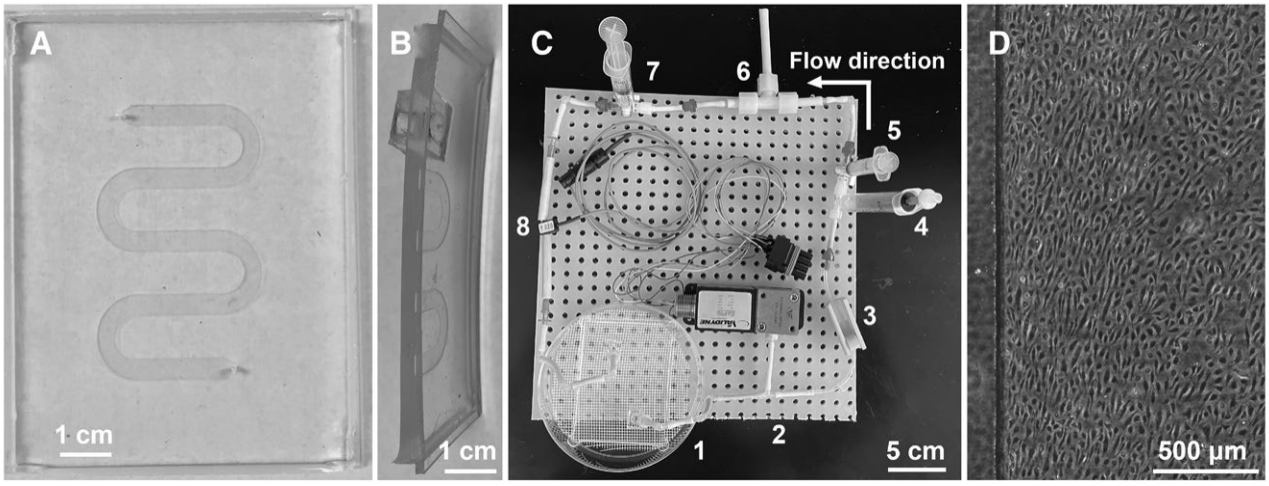

Figure 1. A and B: Front and cross-sectional view of polydimethylsiloxane (PDMS) microfluidic device functioning as vascular pulse perfusion model (VPPM) cell culture chamber. C: VPPM setup and essential components: (1) cell culture chamber, (2) electronic pressure sensor (Validyne Engineering, USA), (3) Keck-tubing clamp (Duran Group, Switzerland) to provide fluidic resistance that increases baseline pressure, (4) syringe to hold perfused cell culture medium with attached air filter, (5) syringe with three-way valve to inject medium into the closed-loop, (6) custom pumping component containing flexible Penrose tubing, (7) syringe to provide air compliance, (8) electronic flow sensor (Transonic Systems Inc., USA). D: Microscopy imaging (4X) of seeded human aortic endothelial cells (HAECs) within VPPM after 72 hours of culture under pulsatile flow. Although not reported, similar cell phenotype was observed with diminished pulsatility condition.

Gunasekar	02/1/23	4 Color Fig(s):0	16:59	Art: ASAIO-22259

its mold, both components were treated with oxygen plasma (Plasma Cleaner PDC-001, Harrick Plasma, USA), quickly pressed together, and heated on a hotplate at 100°C for 5 min to ensure irreversible bonding and hermetic sealing. The final product was tested in the loop setup for leaks or defects before sterilization and cell seeding. To prevent the bottom membrane from sticking to other surfaces, the assembled microfluidic device is stored on top of a nonstick wire mesh throughout use.

Other VPPM essential components are presented in Figure 1C. Before use, all VPPM components were sterilized by either an autoclave or ethylene oxide gas (Anderson Sterilizers AQ6 Inc., USA). VPPM assembly was performed in a sterile environment (1300 Series A2 safety cabinet, Thermo Scientific, USA) and transferred to a large incubator (Norlake Scientific, USA) for culture. A pulsatile pneumatic pump (L.B. Engineering, Germany) located outside the incubator drove fluid flow in the VPPM via the pump component. The pneumatic pump pressure and frequency, the VPPM resistance element, and the air compliance were carefully adjusted simultaneously to obtain the desired pressure and flow waveforms for either normal F2 pulsatile flow or flow with diminished pulsatility (Figure 2B). Pressure and flow sensors were connected to LabView for real-time monitoring. Mean cell culture chamber wall shear stress was calculated to be within 10–15 dyne/cm^2 at the target flow rate, and stretch was maintained within 6–10% via regular observation in both conditions. The incubator was operated at 37°C and in a 5% CO_2 environment.

Seeding of Human Endothelial Cells Into Vascular Pulse Pressure Model

Before seeding, a sterile PDMS microfluidic device was coated with 15 µl/ml human fibronectin (Corning, USA) and incubated for at least 30 mins at 37°C to facilitate strong cell attachment. Primary adult human aortic endothelial cells (HAECs) (Cat#: PCS-100-011, ATCC, USA) were cultured using endothelial cell growth medium EGM-2 Bullet Kit (Lonza, USA) at 37°C with 5% CO_2 until reaching the desired confluency.

For seeding, HAECs were dissociated using 0.05% trypsin-EDTA (Gibco, Canada), incubated at 37°C for 4 mins, and the dissociated cells were centrifuged at 200 × *g* for 5 mins. The cell count was confirmed at least 95% viability using a Countess II instrument (Invitrogen, USA). Approximately 4×10^6 cells were seeded into each PDMS microfluidic device by injecting the appropriate volume of cell suspension using sterile syringes attached to a syringe needle. After seeding, a small, sterilized metal stopper was used to seal the outlet to stop fluid flow and prevent the loss of seeded cells. The device was incubated at 37°C with a 5% CO_2 level for 1–2 days until HAECs were evenly distributed to attain a confluent monolayer. Each device was then subjected to either pulsatile or diminished pulsatility for 72 hours. For each *in vitro* VPPM study, approximately 15 ml of EGM-2 cell culture medium, supplemented with 2% Penicillin-Streptomycin (Gibco, USA), was used for perfusion. Cell phenotypes were observed at the end of the experiments, and images were taken using a Nikon Eclipse TE2000-U epifluorescence microscope (Nikon Instrument Inc., USA) (Figure 1C).

Profiling of Biomarkers In Vitro

For sample collection, the cell culture medium from completed VPPM experiments was collected and stored at −80°C until use. For cytokine assays, the vWF level was measured using Human vWF ELISA Kit (Cat #: RAB0556, Sigma-Aldrich, USA), according to protocols designed by the manufacturer. Levels of another 14 different proinflammatory or pro-angiogenic cytokine levels were measured using Human High Sensitivity Discovery Assay (*i.e.*, HDHSTC14) at Eve Technologies Ltd., Canada. All samples were run in duplicates, and average values were used for data analysis.

Profiling of Biomarkers in Continuous-Flow Ventricular Assist Device Patients

Blood samples were collected from patients selected for CF-VAD placement via the ongoing University of Alabama at Birmingham (UAB) IRB protocol #: IRB-300003505 in coordination with the HF unit at UAB hospital. Patient blood was obtained around 1–2 days before CF-VAD placement and monthly following CF-VAD implant using 4 ml blood collection tubes (green-capped, Lithium Heparin coated B.D. Vacutainer) (B.D., Cat # 367884, USA). Blood samples were centrifuged at 960 × *g* for 15 mins at room temperature to harvest plasma. Patient plasma samples were stored in 2 ml Eppendorf tubes at −80°C until use. Patients' personal information was coded to protect their identity and avoid bias in data analysis. The vWF level was measured for cytokine assays using the Human vWF ELISA Kit (Cat #: RAB0556, Sigma-Aldrich, USA) per the manufacturer's protocol. Angiopoietin-2 (ANGPT-2) and 30 other

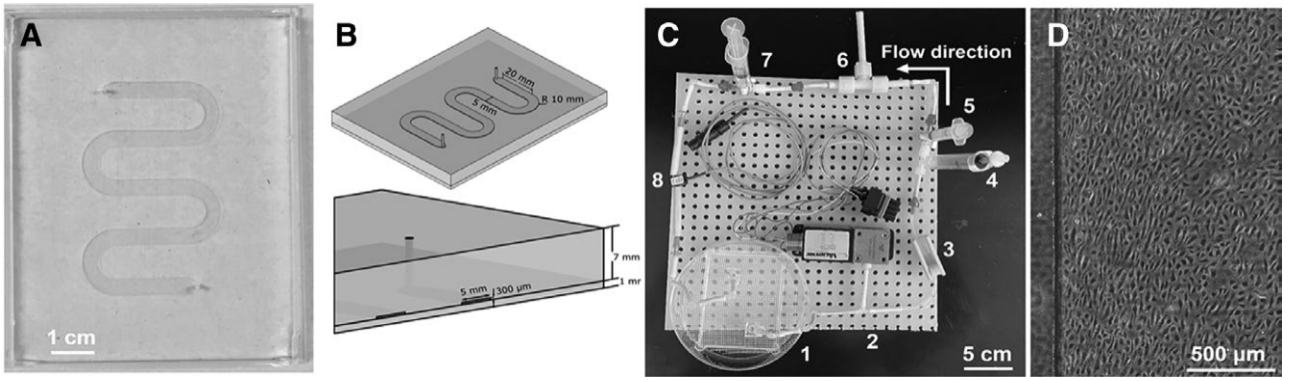

Figure 2. A: Panels of pressure and flow rate waveforms corresponding to vascular pulse perfusion model (VPPM)–generated flows. B: Numerical characteristics of these flows.

Gunasekar	02/1/23	4 Color Fig(s):0	16:59	Art: ASAIO-22259

proinflammatory/proangiogenic cytokine levels were measured using Human High Sensitivity Discovery Assay (HDHSTC14) and Human Angiogenesis & Growth Factor Discovery Assay (HDAGP17) at Eve Technologies Ltd. A detailed list of cytokines for each assay can be found on the company website. All samples were run in duplicates, and average values were used for data analysis.

Data Analysis and Statistical Study

AQ7 Data are represented as mean ± SEM. GraphPad PRISM 9 software was used to perform statistical analysis and prepare reported graphs. The data set was tested for Gaussian normal distribution. For the normal distributed data set, Student's unpaired t-test was used for comparison of *in vitro* data, and Student's paired t-test was used to compare data observed in patient samples. Otherwise, another suitable test was applied, that is, the Wilcoxon matched-pair signed rank test was used for all comparisons among vWF levels in CF-VAD patients. Two-tailed statistical significance was set at $p < 0.05$. In graphs, (*) denotes $p < 0.05$, and (**) denotes $p < 0.01$.

Results

Characterization of Continuous-Flow Ventricular Assist Device–Generated Flows

The VPPM was able to generate pulsatile pressure and flow profiles similar to those under normal blood flow in the arterial system and those seen in arteries of advanced HF patients implanted with CF-VADs (Figure 2A). Specifically, VPPM-generated normal pulsatile-flow present in healthy individuals (referred to as normal pulsatile flow), where physiologic pulse pressure was maintained close to 40 mmHg, and systolic/diastolic pressure was maintained at 120/80 mmHg. VPPM-generated normal pulsatile flow had peak flow rates around 40 ml/min with periods of no flow. In contrast, VPPM-generated flow with diminished pulsatility (referred to as CF-VAD con-
AQ8 tinuous flow) was characterized by a reduced pulse pressure of approximately 5 mmHg and systolic/diastolic pressure maintained at 110/105 mmHg. The flow rate fluctuated between 8 and 18 ml/min. Both conditions were run at the same pumping frequency of 60 bpm. The numerical profiles of both flows are summarized in Figure 2B. Finally, there was a brief period of retrograde flow during valve closure in the VPPM, similar to aortic valve closure found *in vivo*.

Identification of Biomarkers In Vitro

Results from our *in vitro* study using VPPM with the culture of primary adult HAECs suggested that 72 hour diminished pulsatility remarkably affects endothelial production of vWF and ANGPT-2 (Figure 3). Specifically, exposure to continuous flow F3 resulted in a 45% decrease in vWF level from a mean value of 9.80 ± 1.107 ng/ml to 5.32 ± 1.333 ng/ml (n = 5, $p = 0.0327$) and approximately threefold increase of ANGPT-2 from a mean value of 775.29 ± 184.9 pg/ml to 2471.93 ± 635.4 pg/ml (n = 5, $p = 0.0334$) in the perfused cell culture medium. In addition, interleukin-6 (IL-6) and IL-8 decreased slightly from 132.9 ± 18.82 pg/ml to 104.9 ± 58.97 and from 656.1 ± 70.81 pg/ml to 574.2 ± 214.3 pg/ml, respectively, although without statistical significance (n = 3).

Validation of Biomarkers Against Evaluation of Patient Samples

The patient's plasma levels of vWF were also measured using their pre-VAD (referred to as baseline) and post-VAD plasma samples of 1, 2, and 3 months (Figure 4A). Similar to *in vitro*, F4 the evaluation of patient samples suggests that the patient's plasma vWF level decreases approximately 50%, from a mean value of 45.59 ± 8.254 µg/ml at baseline to 22.49 ± 3.85 µg/ml (n = 15, $p = 0.0012$) after 1 month of CF-VAD support (m1 post- AQ9 VAD). Interestingly, this reduced level remained unchanged for additional 2 months, with the mean vWF level of 26.96 ± 5.526 µg/ml after 2 months post-VAD and 23.75 ± 5.754 µg/ml after 3 months post-VAD ($p = 0.068$ for both cases compared with baseline level).

In addition, more than 30 different proinflammatory and proangiogenic cytokines were screened using plasma samples collected 1–2 days before CF-VAD implant for patient baseline and a month after CF-VAD implant for post-VAD soluble factor levels. The results showed that most proinflammatory/

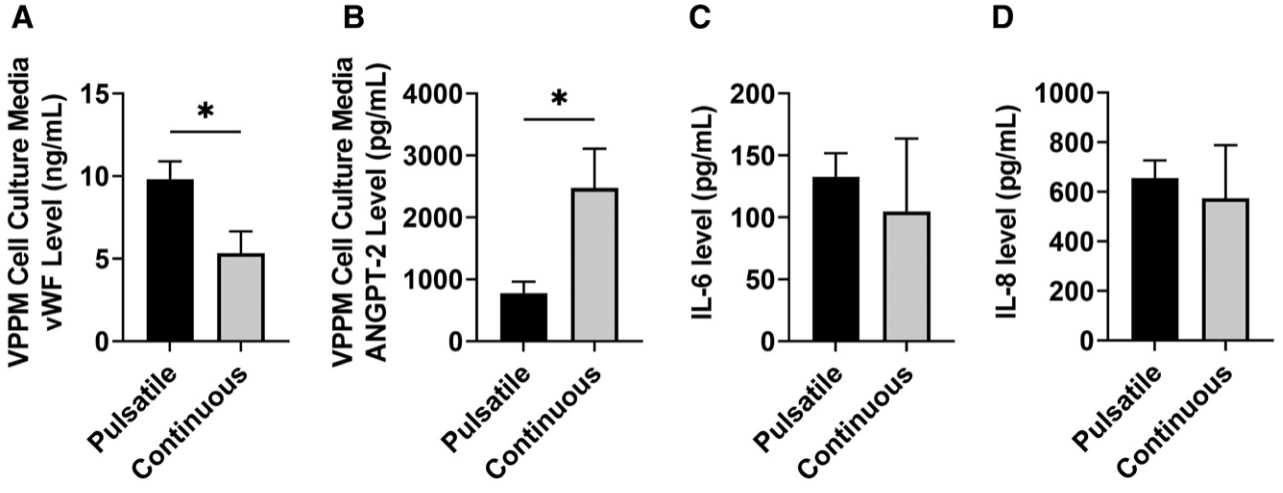

Figure 3. Changes in vascular pulse perfusion model (VPPM) perfused cell culture medium levels of biomarkers.

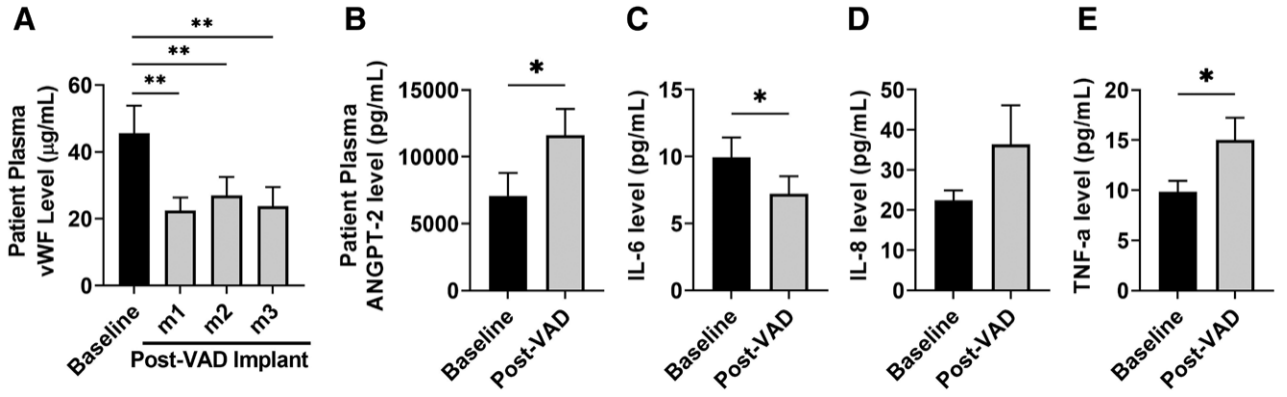

Figure 4. Changes in continuous-flow ventricular assist device (CF-VAD) patient plasma levels of biomarkers.

proangiogenic soluble factors had either insignificant or random changes post-VAD compared to baseline levels. These results were not surprising because CF-VAD patients were often severely ill, on multiple medications, and had comorbidities that could influence their clinical outcomes to an additional major treatment like CF-VAD in unpredictable ways. However, it is noteworthy that there was a statistically significant increase of 64% in patient plasma level changes of ANGPT-2 level from a mean value of 7074±1715 pg/ml to that of 11615±1967 pg/ml (n = 8, p = 0.0376), (Figure 4B) in agreement with *in vitro* results. Patient plasma levels of IL-6 decreased by 27% with statistical significance from 9.93±1.497 pg/ml to 7.224±1.309 pg/ml (n = 8, p = 0.0186), similar to the trend of change seen *in vitro*. Patient plasma levels of IL-8 increased by 62% from 22.48±2.464 pg/ml to 36.34±9.743 pg/ml, but without statistical significance (n = 8, p = 0.1148) (Figure 4C). Interestingly, patient plasma levels of TNF-α also increased substantially by 52% from 9.834±1.104 pg/ml to 15.01±2.236 pg/ml (n = 8, p = 0.0220), making it another candidate biomarker for identifying CF-VAD patients at high risk of nonsurgical bleeding. However, the level of TNF-a was too small within VPPM for detectable results and *in vitro* validation, possibly because TNF-α is mainly produced by immune cells (*e.g.*, macrophages, T lymphocytes, and natural killer cells) and not by aortic endothelial cells.

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Discussion and Conclusion

There is growing evidence that the diminished pulsatil-ity associated with CF-VAD support plays a critical role in the development of nonsurgical bleeding. The arterial endothelium represents the primary location where the effects of diminished pulsatility manifest on the arterial endothelial cells that line the arterial vessels. Our prior work demonstrated that diminished pulsatility leads to dysfunction of HAECs and triggers acute proinflammatory/proangiogenic responses.[20–22] This study aimed to profile and identify endothelial vWF and proinflammatory/proangiogenic soluble factors as pulsatility-sensitive biomarkers. The VPPM allows us to screen for these biomarkers in physiologically relevant and highly controlled conditions without other confounding factors. The comparison of soluble factor level changes *in vitro* and patient plasma samples establishes meaningful correlations that helped validate VPPM-identified biomarkers as reliable and sensitive to pulsatility.

Our study results identified ANGPT-2 and vWF as highly sensitive to pulsatility and IL-6, IL-8, and TNF- α as other promising biomarker candidates. Furthermore, published literature supports our clinical observations regarding the identified cytokines. Concerning proinflammatory/proangiogenic responses, ANGPT-2, TNF- α, and IL-8 were reported to be significantly higher in patients on CF-VAD support compared with their healthy baseline levels, patients on PF-VAD, or patients having a heart transplant.[23–26] Elevated levels of ANGPT-2 and TNF-α were also associated with AVMs and bleeding events not limited to VADs.[12,27,28] While prior studies have not been conclusive regarding the changes in plasma vWF levels, our study demonstrates a consistent and significant decrease in plasma vWF levels following CF-VAD placement.[15,29] In addition, direct visualization studies of vWF suggest that diminished pulsatility, as seen with CF-VAD, significantly increased the unraveling and elongation of surface-bound vWF molecules (analogous to endothelial cell-bound vWF) (p < 0.05), which can potentially enhance the cleavage of vWF into low molecular weight (MW) multimers.[30] Additional evidence suggests that loss of pulsatility may reduce the production of vWF,[31] and loss of high MW vWF also caused endothelial dysfunction.[32,33]

Before translating any promising treatment strategies to mitigate the loss of pulsatility-related adverse events with CF-VAD support, an extensive evaluation of the physiological effects of artificial pulsatility on the circulatory system needs to be accomplished using relevant models. Unfortunately, small animal models do not faithfully simulate human disease and are not large enough to accommodate CF-VADs. Large animal models (calves and pigs) are comparable in size and anatomical structure and can be used to establish stable and irreversible HF.[34] However, nonsurgical bleeding events associated with diminished pulsatility in CF-VAD support are rarely seen in these models. This is likely because they do not mimic the effects of aging seen in human VAD recipients. Furthermore, large quadrupedal animals (calf, pigs, sheep, etc.) have vascular impedances that are markedly different from bipedal humans.[35] Since the arterial endothelium is the major transducer of pulsatility in the body, a vascular cell culture model constructed using human endothelial cells could serve as a viable and cost-effective model to accomplish an early-stage evaluation of promising treatments. However, to truly mimic the effects of diminished pulsatility *in vitro*, arterial endothelial cells must be evaluated in an environment that mimics

critical aspects of flow pulsatility including pulse pressure, shear stress, and stretch. Arterial endothelial cells rely on these mechanical stress signals seen during hemodynamic loading and unloading as feedback mechanisms to maintain normal homeostasis. To overcome shortcomings with available systems for the culture of endothelial cells, the VPPM platform stimulates endothelial cells under realistic mechanical loading conditions and *in vivo*-like pressure, compliance, and flow patterns of vessels. The VPPM cell culture channel represents a circumferential section of the aortic wall and accurately represents the physiological values for flow, pressure, stretch, and shear stress in the aortic section.[17] Notably, the VPPM allows the *in vitro* study of diminished pulsatility in highly controlled conditions to evaluate potential treatments like flow modulation or therapeutic drugs. Limitations with animal models can possibly be overcome via the VPPM platform with *in vitro* culture of primary human adult endothelial cells and represent a viable alternative for testing different treatment options to mitigate the adverse events associated with loss of pulsatility. This study is particularly significant as it identified changes in biomarkers (ANGPT-2 and vWF) within the VPPM and also mirrored changes seen in patients before and post-CF-VAD placement. Ultimately, they can potentially serve as biomarkers to evaluate pulsatility effects and to identify and optimize promising treatments before clinical use.

Limitations of our study include a small *in vitro* sample size (3–5 replications) and the duration of the *in vitro* study (72 hours). This possibly prevented us from observing statistical significance with biomarker candidates that showed consistent trends with diminished pulsatility (a type II statistical error). ANGPT-2 analysis was included at a later time point in the protocol compared with vWF analysis. Thus, despite demonstrating statistical significance, there were fewer patients with ANGPT-2 analysis. Clinical reports from other groups do not follow the same trend of declining vWF observed in our patient cohort.[36–38] This is potentially due to variations in CF-VAD support level affecting pulsatility and the use of ELISA in our study to quantify levels of vWF, whereas the other clinical studies used different functional assays. Despite these limitations, the VPPM provides a controlled, high throughput platform that can enable the identification of biomarkers, investigate mechanisms, and evaluate future therapies.

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