Elevated Angiopoietin-2 Level in Patients With Continuous-Flow Left Ventricular Assist Devices Leads to Altered Angiogenesis and Is Associated With Higher Nonsurgical Bleeding

BACKGROUND: Nonsurgical bleeding is the most common adverse event in patients with continuous-flow left ventricular assist devices (LVADs) and is caused by arteriovenous malformations. We hypothesized that deregulation of an angiogenic factor, angiopoietin-2 (Ang-2), in patients with LVADs leads to increased angiogenesis and higher nonsurgical bleeding.

METHODS: Ang-2 and thrombin levels were measured by ELISA and Western blotting, respectively, in blood samples from 101 patients with heart failure, LVAD, or orthotopic heart transplantation. Ang-2 expression in endothelial biopsy was quantified by immunofluorescence. Angiogenesis was determined by in vitro tube formation from serum from each patient with or without Ang-2–blocking antibody. Ang-2 gene expression was measured by reverse transcription–polymerase chain reaction in endothelial cells incubated with plasma from each patient with or without the thrombin receptor blocker vorapaxar.

RESULTS: Compared with patients with heart failure or those with orthotopic heart transplantation, serum levels and endothelial expression of Ang-2 were higher in LVAD patients (P=0.001 and P<0.001, respectively). This corresponded to an increased angiogenic potential of serum from patients with LVADs (P<0.001), which was normalized with Ang-2 blockade. Furthermore, plasma from LVAD patients contained higher amounts of thrombin (P=0.003), which was associated with activation of the contact coagulation system. Plasma from LVAD patients induced more Ang-2 gene expression in endothelial cells (P<0.001), which was reduced with thrombin receptor blockade (P=0.013). LVAD patients with Ang-2 levels above the mean (12.32 ng/mL) had more nonsurgical bleeding events compared with patients with Ang-2 levels below the mean (P=0.003).

CONCLUSIONS: Our findings indicate that thrombin-induced Ang-2 expression in LVAD patients leads to increased angiogenesis in vitro and may be associated with higher nonsurgical bleeding events. Ang-2 therefore may contribute to arteriovenous malformation formation and subsequent bleeding in LVAD patients.

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Clinical Perspective

What Is New?

- Angiodysplasia leading to nonsurgical bleeding (NSB) is a common complication in patients with left ventricular assist devices (LVADs); however, its cause remains unknown. We found that angiopoietin-2 (Ang-2), a potent angiogenic mediator, is elevated in patients with LVADs. Elevated levels of Ang-2 in these patients increased angiogenesis in vitro and were associated with bleeding events.
- Furthermore, we found that increased thrombin levels in LVAD patients were associated with elevated Ang-2 levels. Our findings therefore suggest that high levels of thrombin induce endothelial Ang-2 expression, which may contribute to angiodysplasia and NSB in LVAD patients.

What Are the Clinical Implications?

- Our findings link the activation of the coagulation system in LVAD patients with altered angiogenesis. This may contribute to the development of angiodysplasia and NSB. Because several Ang-2 inhibitors are currently being developed for the treatment of cancer, these inhibitors might also be useful in preventing or treating LVAD-related angiodysplasia and NSB. In addition, inhibition of the contact coagulation system, including factor XII, may reduce thrombin generation and Ang-2 expression in LVAD patients.
- Our findings suggest that further clinical studies using Ang-2 and factor XII inhibitors may show therapeutic benefits in preventing NSB in LVAD patients.

In the last decade, left ventricular assist devices (LVADs) have emerged as an important therapeutic option for supporting patients with advanced heart failure (HF) both as a bridge to heart transplantation and as destination therapy. Although the use of LVADs has improved survival and quality of life for patients with advanced HF, nonsurgical bleeding (NSB), defined as bleeding at a nonoperative site, commonly complicates the post-LVAD course and frequently leads to increased morbidity and mortality.[1] Arteriovenous malformations (AVMs) in the gastrointestinal tract, nasopharynx, brain, and other tissues are the most common cause of NSB in patients with continuous-flow LVADs,[2] although the reason remains unknown. Interestingly, myocardial capillary density has been shown to be increased after LVAD implantation.[3] This finding, coupled with the development of AVMs, suggests that LVAD implantation may be associated with deregulated angiogenesis at multiple sites.

The angiopoietins (Ang-1 and Ang-2) are a family of molecules that promote angiogenesis. Ang-1 is synthesized by perivascular cells and acts as an agonist of Tie-2, a receptor tyrosine kinase expressed on the surface of endothelial cells. Ang-1 promotes vessel maturity and stability and promotes normal vessel growth in conjunction with vascular endothelial growth factor (VEGF).[4] In contrast, Ang-2 is synthesized exclusively by endothelial cells and is stored with von Willebrand factor within Weibel-Palade bodies.[5] On exocytosis from endothelial cells, Ang-2 also binds to Tie-2, thereby competitively inhibiting Ang-1, and, in concert with VEGF, promotes altered vessel growth.[4] Although both Ang-1 and Ang-2 act in concert with VEGF to promote angiogenesis, Ang-1 promotes normal vessel growth and Ang-2 promotes abnormal growth associated with vascular destabilization and inflammation.[4,6,7] Ang-2–overexpressing mice develop dilated, redundant, tortuous capillaries and lesions of the alimentary tract.[8] Indeed, increased expression of Ang-2 with a corresponding decrease in Ang-1 is associated with vascular malformations[9] and gastrointestinal angiodysplasia.[10]

Activation of the thrombin receptor PAR-1 (protease-activated receptor-1) on the endothelial cell surface promotes Ang-2 expression and release from endothelial cells.[11–15] Prior studies have suggested that plasma levels of thrombin may be elevated in patients with LVADs.[16–18] Given the known relationship between thrombin-dependent PAR-1 activation and Ang-2 expression and release, we hypothesized that thrombin-induced Ang-2 overexpression in patients with LVADs may promote altered blood vessel growth. This study aims to evaluate the expression of Ang-2 in LVAD patients and to assess its correlation with neovascularization and NSB.

METHODS

Study Participants

A cross-sectional study was performed. The study included 3 groups of patients: adult patients supported with an LVAD (Thoratec HeartMate II or Heartware HVAD) at least 30 days after implantation, HF patients with reduced ejection fraction (defined as a left ventricular ejection fraction <40%) without an LVAD, and patients with history of orthotopic heart transplantation (OHT) at least 30 days after transplantation. Patients were recruited in the outpatient cardiology clinic or in the cardiac catheterization laboratory and were clinically stable at the time of enrollment. Patients were excluded from the study if they had decompensated HF, active cancer within 1 year, untreated hypoxic conditions, acute thrombosis within 6 months, severe renal disease defined as an estimated glomerular filtration rate <30 $mL \cdot min^{-1} \cdot 1.73\ m^{-2}$, or acute illness of any kind. Patients treated with direct thrombin inhibitors or factor Xa inhibitors at the time of screening were also excluded to avoid confounding effects of these drugs on the measured activity of thrombin and associated biomarkers. Clinical information was obtained from the medical record. LVAD parameters were obtained from the LVAD control module. Blood pressure was measured with an automated blood pressure cuff, which has recently been reported as the most accurate method for measuring blood pressure in patients with LVADs.[19] All patients were studied in the fasting state. The study protocol was approved by the University of Chicago Institutional Review Board, and all participants provided written informed consent.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown on T-75 flasks (Falcon) with endothelial growth medium-2 (Lonza) under standard conditions (37°C, 5% CO_2). Cells were grown to 70% confluence, washed in phosphate-buffered saline solution (PBS), trypsinized, and passaged. For all experiments, HUVECs were used for experiments before passage 7.

Measurement of Circulating Biomarkers

Peripheral venous blood was obtained by venipuncture and collected in vacutainer tubes (BD Bioscience) containing EDTA, sodium heparin, sodium citrate, or silica clot activator. Samples were immediately centrifuged at 2000*g* for 20 minutes at 4°C. The plasma and serum fractions were collected, divided, and frozen at −80°C for future analysis. VEGF and Ang-1 levels were measured in platelet-poor plasma (EDTA), and Ang-2 and soluble Tie-2 levels were measured in serum by ELISA (R&D Systems). Because certain phlebotomy techniques could induce artefactual thrombin formation, both thrombin and prothrombin were measured in plasma (EDTA) drawn through an 18-gauge butterfly needle from the antecubital vein. Thrombin and prothrombin levels were then determined by Western blot using rabbit anti-human thrombin/prothrombin primary antibody (1:1000 dilution; Abcam) and horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (1:2500 dilution; Bio-Rad). Factor XIIa and XIa levels were measured by Western blot using rabbit anti-human factor XII C-terminal antibody (1:1000 dilution; Abcam) and mouse anti-human factor XI light-chain antibody (1 μg/mL dilution; R&D Systems), respectively, and horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibody (1:2500 dilution; Bio-Rad). Blot images were analyzed by use of a ChemiDoc imaging system with Bio-Rad Image Laboratory 5.1 software.

Harvesting Endothelial Cells From Patients

Vena caval endothelial cells were obtained from guidewires used during right-sided heart catheterization as described elsewhere.[20–24] Briefly, central venous access was obtained with a modified Seldinger technique, and a 6F venous sheath was placed in the femoral vein or internal jugular vein over a 0.035-in J wire (Arrow International). The J wire was advanced as far as possible through the vena cava, and endothelial cells were collected by incidental abrasion with the vessel wall. Endothelial cells were recovered from the wire by centrifugation in a dissociation buffer and plated on poly-l-lysine–coated microscope slides (Sigma). Cells were fixed immediately in 4% paraformaldehyde, washed in PBS, dried, and stored at −80°C for further processing.

Assessment of Protein Expression by Quantitative Immunofluorescence

Samples were analyzed as described.[23] Briefly, fixed endothelial cells were stained with primary antibodies against Ang-2 (1:150 dilution; R&D Systems) and von Willebrand factor (1:300 dilution; Dako), followed by fluorescent-labeled secondary antibodies (1:200 dilution; Invitrogen), and then mounted under glass coverslips with Vectashield containing DAPI for nuclear identification (Vector Laboratories). For each batch of patient-derived cells, a control slide of cultured HUVECs taken from a single passage was stained contemporaneously. Slides were imaged on an Olympus BX41 fluorescent microscope at ×20 magnification and analyzed with Image J software.[25] Fluorescent intensity of Ang-2 was quantified in 20 random cells from each patient, and the results were averaged. Fluorescent intensity for each patient sample was then normalized to the intensity of the HUVEC control slide for the corresponding batch to correct for batch-to-batch variability in staining. Intensity is expressed in arbitrary units (AU) calculated by dividing the average fluorescent intensity from the patient sample by the average fluorescent intensity of the HUVEC control sample and multiplying by 100. Quantifications were performed by technicians who were blinded to patient identity and cohort status.

Assessment of Angiogenic Potential of Patients' Serum

The 24-well cell culture plates (Falcon) were coated with Matrigel (Corning Life Sciences) and allowed to solidify at 37°C for 1 hour. Cultured HUVECs were then washed with PBS, trypsinized, centrifuged, and resuspended in a mixture of 50% serum from individual patients with HF, LVAD, or OHT and 50% Endothelial Basal Medium-2 (Lonza) with growth factor additives such that the final concentration of each exogenous growth factor in the serum/endothelial basal medium-2 mixture was equal to that in endothelial growth medium-2 (Lonza). This mixture containing 200 000 HUVECs was then gently pipetted into the Matrigel-coated wells and incubated for 18 hours under standard conditions in the presence or absence of an Ang-2–blocking antibody (150 ng/mL, azide-free mouse/anti-human Ang-2, Adipogen), which specifically inhibits binding of Ang-2 to Tie-2 but does not affect binding of Ang-1 to Tie-2. Cultures were then stained with Calcein (Corning) to improve microtube visibility. Microtube formation was assessed by microscopy as described.[26] Briefly, total tube number in a low-power field was quantified (5 fields per well were averaged). All quantifications were performed by technicians who were blinded to patient identity and cohort status.

Assessment of the Effect of Thrombin on Ang-2 Expression

Plasma was obtained from an antecubital vein through an 18-gauge butterfly needle and anticoagulated with fondaparinux (1 μg/mL). Samples were gently mixed, centrifuged at 2000*g* for 20 minutes at 4°C, divided, and frozen at −80°C. HUVECs were grown to 70% confluence on 6-well plates (Falcon) under standard conditions and serum-starved overnight in endothelial basal medium-2 supplemented with 2% fetal bovine serum (Life Technologies) but devoid of supplemental growth factors. Cultures were then incubated with the thrombin receptor blocker vorapaxar (100 μg/mL, Adooq Bioscience) or vehicle for 2 hours. During this time, the frozen plasma samples obtained previously were warmed to 37°C, mixed, and centrifuged at 2000*g* for 10 minutes. The plasma sample from each patient was then divided and mixed with either vorapaxar (100 μg/mL in PBS) or an equal amount of PBS alone. After 2 hours, the cell culture media was carefully aspirated from each well, and the plasma samples with or without vorapaxar were added to each well. Cultures were then incubated for an

additional 4 hours under standard conditions. After incubation, the plasma was aspirated, and the cultures were washed with PBS. RNA was isolated with a PureLink RNA Mini Kit (Life Technologies), and Ang-2 gene expression was quantified by reverse transcription–polymerase chain reaction.

Statistical Analyses

Statistical analyses were performed with SPSS version 23.0. Continuous variables such as biomarkers were compared among groups by use of the Kruskal-Wallis test, followed by pairwise post hoc comparisons with the Mann-Whitney U test with Bonferroni adjustment when the omnibus test indicated a significant difference among the cohorts. Treatment conditions were compared within groups with the Wilcoxon signed-rank test. Categorical variables such as bleeding events were compared by use of the Fisher exact test. The Pearson correlation was used to evaluate the relationship between serum levels of Ang-2 and endothelial tube formation on Matrigel. Clinical characteristics were compared by use of ANOVA, the Student *t* test for continuous variables, or the Pearson χ^2 test for categorical variables as appropriate. Ordinal variables such as New York Heart Association HF class were compared by use of the Wilcoxon rank-sum test. Data are presented as mean±SD unless otherwise indicated. A 2-sided value of $P<0.05$ was considered statistically significant.

RESULTS

We enrolled 32 patients with HF, 44 patients with LVADs, and 25 patients with OHT. Clinical characteristics are shown in the Table; subgroups are shown in Tables I through III in the online-only Data Supplement. All groups were similar in age, sex, race, and renal function. As expected, the New York Heart Association HF class was higher (worse) for the HF group compared with the LVAD group. Both the LVAD and OHT groups were studied ≈300 days after implantation. Among the LVAD cohort, 32 patients with a Thoratec HeartMate II and 12 patients with a Heartware HVAD were studied. LVAD flow (HeartMate II, 5.4±1.2 L/min; HVAD, 4.6±1.3 L/min; $P=0.077$), C-reactive protein (HeartMate II, 77.9±44.8 mg/dL; HVAD, 39.2±37.5 mg/dL; $P=0.192$), and lactate dehydrogenase (HeartMate II, 386.7±206.0 U/L; HVAD, 309.3±205.0 U/L; $P=0.272$) were similar between both groups. Pulse pressure was slightly higher in the HVAD group compared with the HeartMate II group (35.17±12.50 versus 27.86±5.64 mm Hg; $P=0.008$). Mean rotor speed was 9115.5±389.3 rpm for patients with a HeartMate II and 2746.7±135.7 rpm for patients with an HVAD. The pulsatility index for HeartMate II patients was 5.6±1.2.

Elevated Circulating Ang-2 and Associated Biomarkers in Patients With LVADs

To evaluate the circulating levels of Ang-2, Ang-1, soluble Tie-2, and VEGF in patients with and without LVADs, we measured serum levels of Ang-2 and soluble Tie-2 and platelet-poor plasma levels of Ang-1 and VEGF by ELISA (Figure 1A–1D). Notably, Ang-2 was higher in patients with LVADs compared with patients with HF or OHT (12.32±9.57, 5.24±2.98, and 4.39±2.00 ng/mL, respectively; omnibus $P=0.001$; HF versus LVAD, $P=0.012$; LVAD versus OHT, $P=0.003$). Soluble Tie-2 was similarly elevated in patients with LVADs (LVAD, 22.73±6.85 ng/mL; HF, 18.97±4.39 ng/mL; and OHT, 16.05±4.92 ng/mL; omnibus $P=0.004$; HF versus LVAD, $P=0.213$; LVAD versus OHT, $P=0.004$), possibly as a result of receptor shedding in response to the action of Ang-2. In contrast, Ang-1 trended lower in patients with LVADs compared with HF (3.73±3.66 and 6.94±6.39 ng/mL respectively; $P=0.055$) and the Ang-1/Ang-2 ratio was lower in patients with LVADs. Interestingly, VEGF was not significantly different in patients with LVADs or HF but trended lower in patients with OHT (147.17±185.57, 181.77±182.46, and 50.48±33.17 pg/mL, respectively; omnibus $P=0.191$). This finding is consistent with prior reports that VEGF is not significantly different in patients with LVADs and patients with HF[27] but is decreased after OHT.[28] These findings demonstrate a shift in Tie-2 regulation from Ang-1 in patients with HF to Ang-2 in patients with LVADs in the presence of continued overexpression of VEGF, a constellation that favors abnormal angiogenesis.[7,29–31] In subset analysis, Ang-2 was significantly higher in LVAD patients with HVAD compared with those with HeartMate II without a significant difference in the other biomarkers measured (Table IV in the online-only Data Supplement). No significant relationship was noted between the length of LVAD support and any biomarker tested (Table V in the online-only Data Supplement). The relationships between biomarkers and warfarin or antiplatelets were challenging to interpret because of the extremely small patient numbers in the subgroups (Tables VIa–VId in the online-only Data Supplement). Levels of VEGF appeared to be higher in LVAD patients whose aortic valve opened with every beat, whereas no relationship was seen between aortic valve opening and the other biomarkers (Table VIIa in the online-only Data Supplement).

Elevated Ang-2 Expression in Freshly Isolated Endothelial Cells From Patients With LVADs

To investigate the source of the elevated circulating Ang-2 in patients with LVADs, we analyzed freshly isolated vena caval endothelial cells from patients with HF, LVAD, or OHT using quantitative immunofluorescence. Consistent with our finding of elevated circulating Ang-2 in blood of patients with LVADs, Ang-2 protein expression in freshly isolated endothelial cells was also higher in patients with LVADs compared with patients with HF or OHT (52.4±19.9, 24.2±10.2, and 35.0±11.2 AU; omnibus $P<0.001$; HF versus LVAD, $P<0.001$; LVAD versus OHT,

Table. Clinical Characteristics

	HF Patients	LVAD Patients	OHT Patients	*P* Value
Participants, n	32	44	25	
Age, y	62.8±11.9	58.8±10.7	54.1±10.9	0.016
Female, %	31	27	24	0.829
Black race, %	34	41	32	0.422
Left ventricular ejection fraction, %	27.3±9.1	…	59.6±8.4	<0.001
Time after implantation (median), d	…	295.0±479.2	311.0±1257.9	0.100
BMI, kg/m^2	32.7±13.9	30.7±7.6	28.1±4.7	0.217
eGFR, mL·min^{-1}·1.73 m^{-2}	68.7±20.6	59.6±24.5	65.8±21.9	0.208
Dilated cardiomyopathy, %	53	59	44	0.482
Ischemic cardiomyopathy, %	41	43	40	0.959
Myocarditis, %	0	2	12	0.052
Hypertension, %	56	46	52	0.640
Diabetes mellitus, %	22	36	48	0.115
Dyslipidemia, %	50	50	56	0.873
NYHA HF class, %				<0.001
1	3	9	…	
2	22	70	…	
3	69	21	…	
4	0	0	…	
Heart rate, bpm	78.1±17.5	83.0±16.9	95.1±11.8	0.001
Mean arterial pressure, mm Hg	88.3±14.7	84.4±17.6	96.2±11.1	0.011
Pulse pressure, mm Hg	49.6±15.7	29.8±8.8	47.0±9.7	<0.001
Hemoglobin, mg/dL	12.7±1.7	11.5±1.7	12.8±1.7	0.002
B-type natriuretic peptide, ng/L	3025.3±3492.0	2345.6±1996.1	…	0.371
Total cholesterol, mg/dL	173.0±55.2	135.4±47.3	166.9±35.8	0.010
HDL, mg/dL	51.9±24.3	34.3±14.1	46.2±14.8	0.003
LDL, mg/dL	95.4±44.0	74.9±35.1	93.3±29.4	0.096
INR	1.6±0.7	1.8±0.5	1.1±0.1	<0.001
Platelet count, n/μL	225.3±63.2	223.6±71.7	193.0±72.2	0.150
Statin, %	44	59	96	<0.001
Warfarin, %	44	93	4	<0.001
ACE-I/ARB, %	84	46	12	<0.001
Antiplatelets, %	53	90	52	<0.001

ACE-I indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HF, heart failure; INR, international normalized ratio; LDL, low-density lipoprotein; LVAD, left ventricular assist device; NYHA, New York Heart Association; and OHT, orthotopic heart transplantation.

P=0.029; Figure 2). These findings suggest that overexpression of Ang-2 in the endothelium may be responsible for the elevated circulating Ang-2 levels in patients with LVADs.

Elevated Ang-2 in Serum From Patients With LVADs Induces Angiogenesis

Previous studies have shown that Ang-2 increases endothelial tube formation on Matrigel.[32] To investigate wheth-

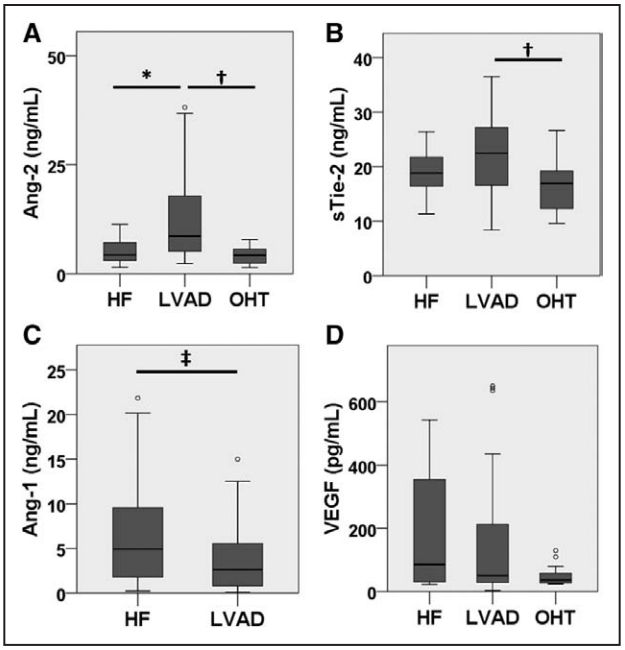

Figure 1. Altered blood levels of angiogenic proteins in patients with left ventricular assist devices (LVADs). Blood levels of angiopoietin (Ang)-1, Ang-2, vascular endothelial growth factor (VEGF), and Tie-2 were measured by ELISA in patients with heart failure (HF), LVAD, or orthotopic heart transplantation (OHT; n=17, 38, and 14, repsectively). **A**, Ang-2 was significantly higher in LVAD patients compared with HF and OHT patients. **B**, Soluble Tie-2 (sTie-2) was significantly higher in LVAD patients compared with OHT patients and increased nonsignificantly compared with HF patients. **C**, Ang-1 trended lower in LVAD patients compared with HF parents. **D**, Plasma VEGF remained elevated in patients with LVADs compared with patients with HF but trended lower in patients with OHT. **P*<0.05. †*P*<0.01. ‡*P*=0.055.

er the elevated Ang-2 in serum from patients with LVADs could induce endothelial tube formation, we incubated HUVECs grown on Matrigel with serum from patients with HF, LVAD, or OHT in the presence or absence of an Ang-2–blocking antibody. Serum from patients with LVADs induced more microtube formation than did serum from patients with HF or OHT (38.15±10.42, 27.44±11.34, and 27.50±6.18 tubes per low-power field, respectively; omnibus *P*<0.001; HF versus LVAD, *P*=0.003; LVAD versus OHT, *P*<0.001; Figure 3A). This effect in the LVAD group was abolished by the Ang-2-blocking antibody (20.71±6.98 tubes per low-power field; *P*<0.001), indicating that an elevated Ang-2 level in the serum from patients with LVADs is the driving factor for the increased microtube formation. No significant difference was observed in the HF or OHT group in response to the Ang-2–blocking antibody (27.14±9.66 and 24.57±7.33 tubes per low-power field, respectively; *P*=NS). Among the LVAD patients, tubule formation correlated strongly with serum Ang-2 level (R^2=0.645, *P*<0.001; Figure 3B).

Elevated Thrombin in Plasma From Patients With LVADs Increases Ang-2 Gene Expression in Endothelium

Previous studies have suggested that plasma levels of thrombin may be elevated in patients with LVADs.[16–18] To confirm this, we measured thrombin and prothrombin in plasma from patients with HF, LVAD, or OHT by Western blot. Indeed, thrombin was elevated in plasma from patients with LVADs compared with HF or OHT (5.88±6.70, 0.93±0.79, and 0.57±0.43 AU, respectively; omnibus *P*=0.003; HF versus LVAD, *P*=0.039; LVAD versus OHT, *P*=0.003; Figure 4A and 4B). Prothrombin, however, was not different among the 3 groups.

To identify the most likely source of the increased thrombin in LVAD patients, we investigated key regulators of the contact coagulation system, specifically factor XIIa and its downstream effector factor XIa, in plasma from patients with HF, LVAD, or OHT by West-

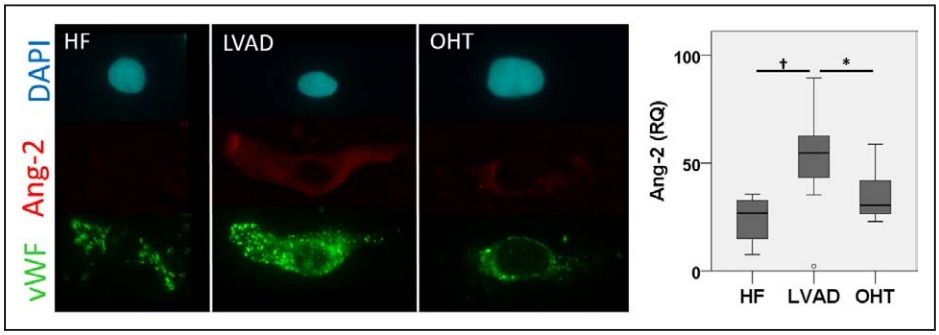

Figure 2. Endothelial angiopoietin-2 (Ang-2) expression is increased in patients with left ventricular assist devices (LVADs). Endothelial biopsy was performed in patients with heart failure (HF), LVADs, or orthotopic heart transplantation (OHT; n=10, 13, and 12, respectively), and endothelial cells were isolated by centrifugation. Cells were plated onto microscope slides and stained with fluorescent-labeled antibodies. Protein content was measured by quantitative immunofluorescence. Endothelial expression of Ang-2 (red) was significantly higher in patients with LVADs compared with patients with HF or OHT. vWF indicates von Willebrand factor. **P*<0.05. †*P*<0.01.

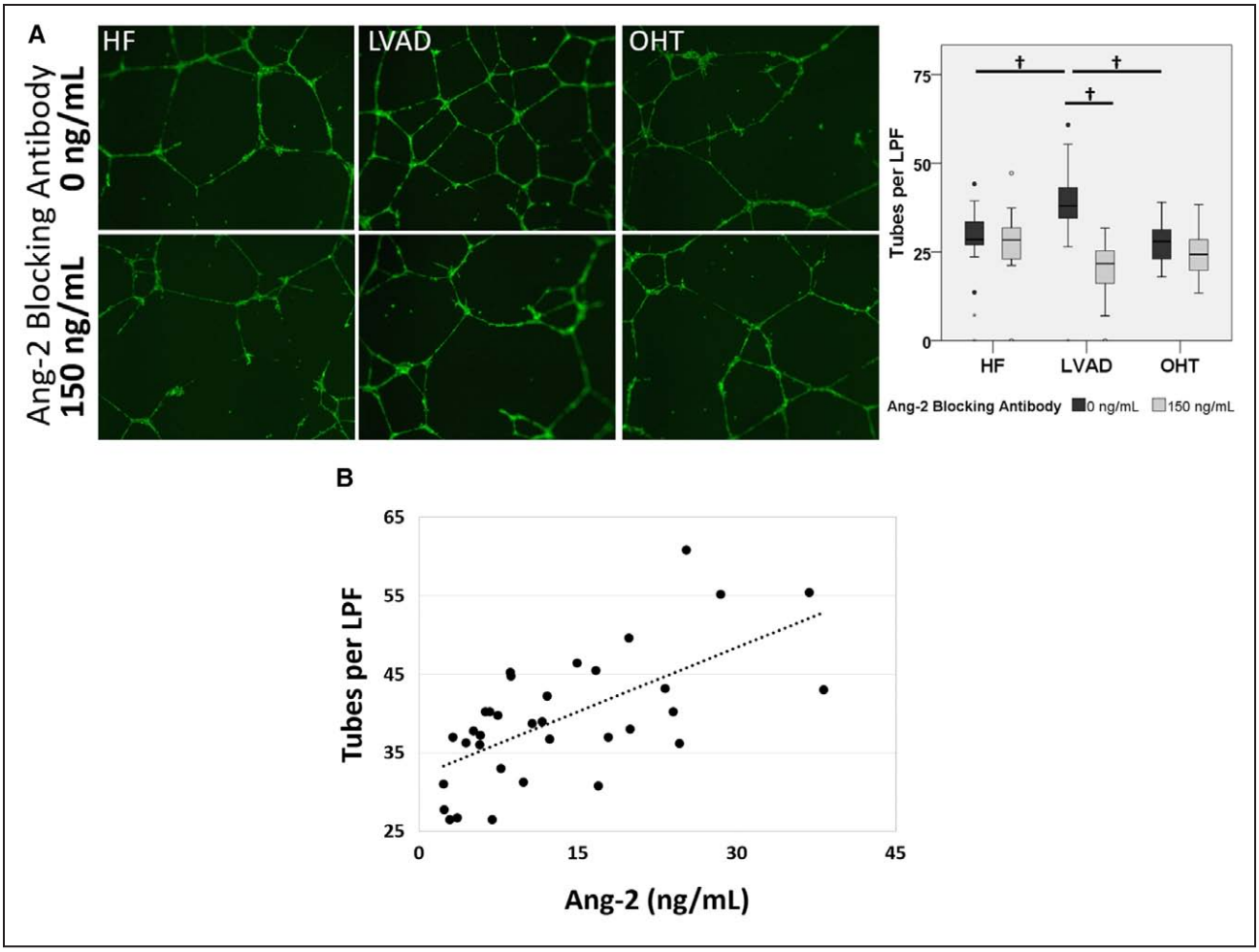

Figure 3. Elevated angiopoietin-2 (Ang-2) in serum from patients with left ventricular assist devices (LVADs) induces angiogenesis in human endothelium.

A, Human umbilical vein endothelial cells were assayed on Matrigel and incubated overnight with serum from patients with heart failure (HF), LVADs, or orthotopic heart transplantation (OHT; n=17, 35, and 14, respectively) in the presence or absence of an Ang-2–blocking antibody. Tubule formation was then quantified visually by an investigator blinded to sample identity. Tubule formation was significantly greater in cultures treated with serum from patients with LVADs compared with control subjects. Cotreatment with an Ang-2–blocking antibody significantly reduced angiogenic growth in cells treated with serum from patients with LVADs but had no significant effect in cells treated with serum from HF or OHT patients. †P<0.01. **B**, Among LVAD patients, tubule formation correlated strongly with serum Ang-2 concentration (R^2=0.645, P<0.001). LPF indicates low-power field.

ern blot. Both factor XIIa (HF, 0.50±0.26 AU; LVAD, 1.01±0.58 AU; and OHT, 0.63±0.28 AU; omnibus P=0.037; HF versus LVAD, P=0.034; LVAD versus OHT, P=0.377) and factor XIa (HF, 0.94±0.47 AU; LVAD, 2.29±1.49 AU; OHT, 0.86±0.21 AU; omnibus P=0.014; HF versus LVAD, P=0.060; LVAD versus OHT, P=0.028) were higher in LVAD patients (Figure 4C–4F), suggesting that activation of the contact coagulation system is a likely source of the increased thrombin seen in LVAD patients.

To evaluate whether the increased thrombin in plasma from patients with LVADs could be responsible for increased Ang-2 gene expression, we incubated HUVECs for 4 hours with plasma anticoagulated with fondaparinux in the presence or absence of vorapaxar, a thrombin receptor (PAR-1) antagonist. The plasma from patients with LVADs induced higher Ang-2 gene expression in the cultured endothelial cells compared with plasma from patients with HF or OHT (1.88±0.63, 0.67±0.15, and 0.53±0.14 relative quantity, respectively; omnibus P<0.001; HF versus LVAD, P=0.003; LVAD versus OHT, P<0.001; Figure 5). This increased Ang-2 expression was significantly reduced with thrombin receptor blockade (0.95±0.22 relative quantity; P=0.013). In contrast, a small but nonsignificant decrease in Ang-2 gene expression in the presence of vorapaxar was noted in endothelial cells receiving plasma from patients with HF or OHT (0.53±0.21 and 0.43±0.12 relative quantity, respectively; P=NS). Taken together, these data suggest that elevated thrombin in plasma from patients with LVADs induces increased endothelial Ang-2 expression.

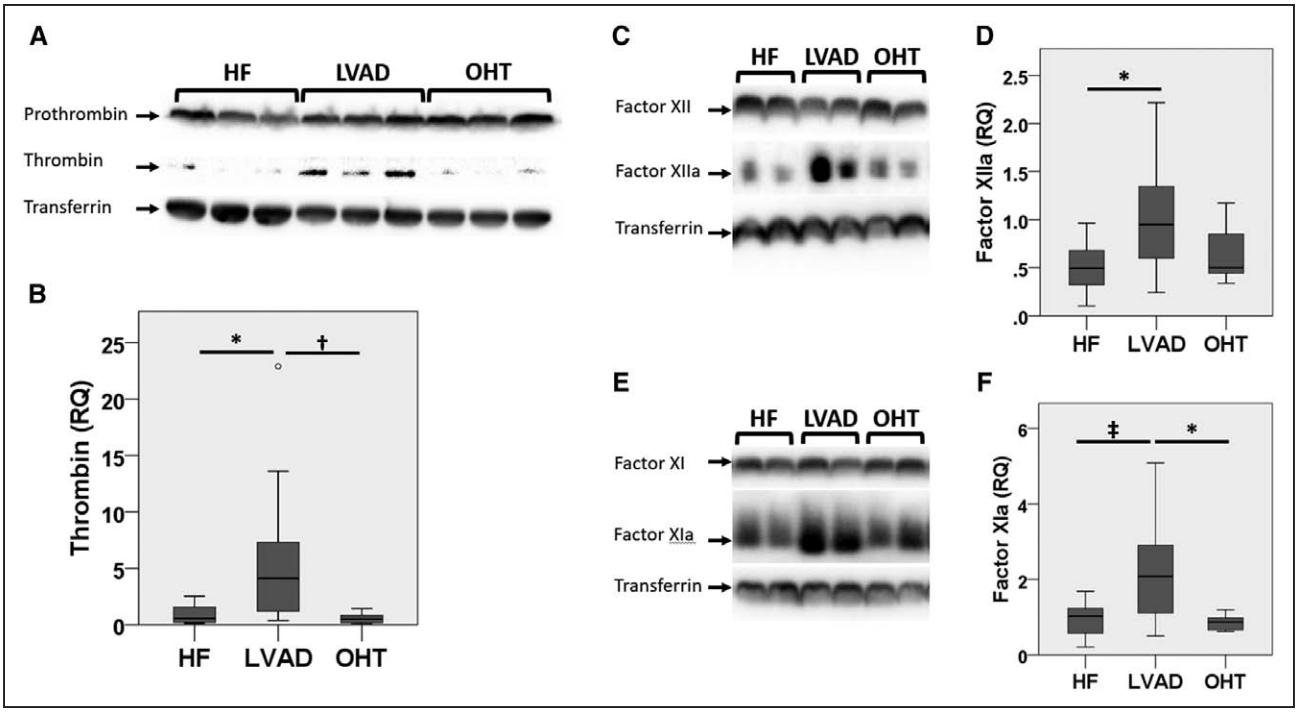

Figure 4. Elevated plasma levels of thrombin in patients with left ventricular assist devices (LVADs). Thrombin was measured by Western blot in plasma from patients with heart failure (HF), LVADs, or orthotopic heart transplantation (OHT; n=14, 13, and 15, respectively). **A** and **B**, Thrombin was significantly higher in plasma from patients with LVADs compared with patients with HF or OHT, whereas prothrombin was unchanged. To investigate a possible source of this elevated thrombin, we measured factors XIIa and XIa by Western blot in plasma from these patients. **C** through **F**, Factors XIIa and XIa were significantly higher in LVAD patients. Taken together, our findings suggest that activation of the contact coagulation system contributes to increased production of thrombin in LVAD patients. **P*<0.05. †*P*<0.01. ‡*P*=0.060.

Elevated Ang-2 in Serum From Patients With LVADs Is Associated With an Increased Risk of NSB Events Within 3 Months of Sample Collection

To investigate the role of serum Ang-2 in predicting NSB events in patients with LVADs, we reviewed the electronic medical records of all patients with LVADs enrolled in the present study for instances of gastrointestinal bleeding, intracranial hemorrhage, or epistaxis. Gastrointestinal bleeding was defined as a report of blood in the stool or vomitus, a finding of hemoccult-positive stool, or a finding of pathological bleeding on endoscopy. Intracranial hemorrhage was defined as a radiographic finding of bleeding within the cranium. Epistaxis was defined as a report of bleeding from the nose or a finding of pathological bleeding within the nasopharynx on endoscopy. Among patients with LVADs and a serum Ang-2 level above the mean of 12.32 ng/mL (n=13), 5 patients had at least 1 NSB event within 3 months of sample collection (4 gastrointestinal bleeds, 1 epistaxis, 0 intracranial hemorrhages), whereas 0 patients with serum Ang-2 level below the mean (n=25) experienced bleeding (*P*=0.003). However, there was no relationship between Ang-2 and NSB at 6 months after sample collection (8 gastrointestinal bleeds, 1 epistaxis, 0 intracranial hemorrhages), suggesting a critical time interval between the elevation of Ang-2 and bleeding events. Ang-2 was significantly higher in patients who experienced bleeding events (27.69±9.74 ng/mL) compared with those who did not (9.99±7.18 ng/mL; *P*<0.001). However, no significant relationship was observed between aortic valve opening and NSB (Table VIIb in the online-only Data Supplement).

DISCUSSION

This is the first study to assess the role of Ang-2 in patients supported with LVADs. We found that patients with LVADs have elevated circulating Ang-2 levels and higher Ang-2 protein expression in the endothelial cells, representing overexpression of Ang-2. The increased Ang-2 levels in patients with LVADs led to increased angiogenesis, which was inhibited by an Ang-2–blocking antibody. Furthermore, LVAD patients have elevated thrombin levels, which stimulate Ang-2 overexpression, and LVAD patients with elevated Ang-2 levels have a higher risk for NSB.

Ang-2 disrupts vital intercellular connections that are associated with vessel maturation[33] and induces endo-

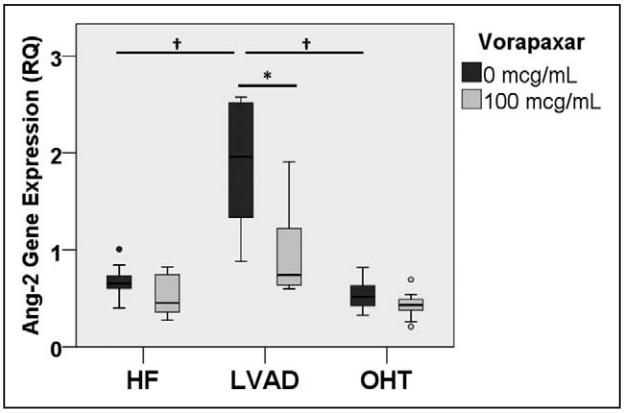

Figure 5. Elevated plasma levels of thrombin in patients with left ventricular assist devices (LVADs) induce endothelial overexpression of angiopoietin-2 (Ang-2). Plasma samples from patients with heart failure (HF), LVADs, or orthotopic heart transplantation (OHT; n=14, n=13, and n=15 respectively) were anticoagulated with fondaparinux to prevent artefactual thrombin generation ex vivo. Cultured human umbilical vein endothelial cells were starved overnight and then incubated with plasma from each patient in the presence or absence of vorapaxar, a thrombin receptor blocker. Ang-2 gene expression was measured by reverse transcription–polymerase chain reaction. Plasma from patients with LVADs induced significantly higher expression of Ang-2 compared with patients with HF or OHT. This effect was normalized with thrombin receptor blockade. *P<0.05. †P<0.01.

thelial inflammation and abnormal angiogenesis,[7,29–31] resulting in tortuous, fragile vessels that are prone to bleeding. Indeed, Ang-2–overexpressing mice develop dilated, tortuous, redundant vessels[8] reminiscent of AVMs in patients with LVADs. Blockade of Ang-2 signaling is currently being investigated in the treatment of various cancers in which neovascularization is associated with accelerated tumor growth.[4] Numerous Ang-2 blockers are currently in commercial development, and many have shown promise in decreasing tumor size.[4] However, it is not known whether Ang-2 blockade can prevent or is an effective therapy for LVAD-related NSB.

In the present study, we found that Ang-2 levels were markedly higher in the blood of patients with LVADs, with even further elevation in patients with HVADs. However, Ang-2 levels were not elevated in patients with OHT, suggesting that the LVAD, not the associated surgery or the increase in cardiac output, is most likely responsible for the conditions leading to Ang-2 overexpression. Notably, the increase in Ang-2 levels in patients with LVADs was mirrored by an increase in soluble Tie-2 (the angiopoietin receptor) and was accompanied by a decrease in Ang-1 levels without a decrease in VEGF levels compared with patients with HF.

Our results provide insight into the potential mechanism and consequences of Ang-2 overexpression in patients with LVADs. Known inducers of Ang-2 secretion include thrombin, catecholamines, and hypoxia. However, catecholamine levels tend to decrease after LVAD implantation,[34] which makes it unlikely that they are the cause of increased Ang-2 expression in these patients. Hypoxia is the best-characterized stimulator of Ang-2 expression, but patients are typically not hypoxic after LVAD implantation. In addition, patients with untreated hypoxia were excluded from our study. In contrast, thrombin has been shown to upregulate Ang-2 expression and release in vitro,[14] and prior studies have suggested that thrombin activity may be increased in patients with LVADs[16,17] as a result of the interaction of coagulation factors with the materials of the LVAD.[18] Specifically, our data suggest that the contact coagulation system (regulated by factors XIIa and XIa) appears to be activated in LVAD patients, which could be a possible source of thrombin generation in these patients. Titanium, the primary material of the LVAD rotor, is known to strongly activate the contact coagulation system,[35] suggesting that the use of alternate materials or the inhibition of the contact coagulation system might improve LVAD hemocompatibility or reduce complications.

In addition to the role of thrombin in converting fibrinogen to fibrin, thrombin is known to activate PAR-1.[12,36] PAR-1 activates the phospholipase-C-β signaling cascade, inducing Weibel-Palade body exocytosis and Ang-2 expression.[15,37] In line with this mechanism, our findings implicate thrombin-induced PAR-1 activation as the most likely mechanism for the increased Ang-2 found in patients with LVADs. The further increase in Ang-2 levels in patients with HVADs is of unclear significance and may represent device-specific factors or patient-specific factors because all patients in our study with an HVAD received the device as part of a bridge-to-transplantation strategy, whereas patients with a HeartMate II used as bridge-to-transplantation or destination therapy were studied.

Most studies showing thrombin activation in patients with LVADs studied first-generation pulsatile LVADs, not the continuous-flow LVADs used in the present study.[16,18] Although one limited study suggested the presence of increased thrombin activity in modern LVAD recipients (through the observation of fibrin split products and thrombin/antithrombin complexes),[17] an increase in circulating active thrombin after LVAD has not previously been shown, and the net effect of modern LVAD implantation on the coagulation system remains controversial. Study of overall thrombin activity in these patients is challenging because all patients with modern LVADs are treated with warfarin. However, patients treated with warfarin still have residual thrombin activity,[38] and patients with LVADs retain a significant risk of LVAD thrombosis despite the use of warfarin.[39] The use of warfarin and antiplatelets remains an unavoidable confounder in our study.

Our study extends our current understanding of the pathogenesis of LVAD-related AVM formation by dem-

onstrating for the first time in patients with LVADs the activation of a pathway well known to cause abnormal blood vessel growth. Previously, the transition from pulsatile flow to continuous flow after LVAD implantation has been hypothesized to drive abnormal vessel growth through an unknown mechanism.[40] Wever-Pinzon and colleagues[41] reported an association between pulsatility and NSB in LVAD recipients. However, in the present study, we found no significant association between aortic valve opening or pulse pressure and NSB events or Ang-2 expression. Although we cannot exclude a role for altered flow conditions in the induction of Ang-2 expression, prior studies have offered conflicting data. Some authors suggest that Ang-2 expression is increased by continuous flow versus pulsatile flow,[42] whereas others suggest the opposite.[43] Furthermore, although the term continuous flow is commonly used to describe flow in patients with LVADs, blood flow in the aorta of LVAD patients is actually quite turbulent because of the high shear forces produced by the LVAD.[44,45] Additionally, flow in the small arterioles, capillaries, and venous system is normally nonpulsatile, and these beds make up an overwhelming majority of the total vascular surface area. In the present study, we show that Ang-2 expression is elevated in the vena caval endothelial cells, where flow is normally nonpulsatile. Therefore, a nonmechanical cause of Ang-2 overexpression is more consistent with known data because the vessels facing altered flow represent a small minority of total vessel area and are distinct from the beds involved in angiogenesis.

Our study has several limitations. Ideally, we would have liked to explore whether pharmacological inhibition of Ang-2 in LVAD patients reduces AVM formation and NSB. Such agents are currently in phase III clinical trials for the treatment of various cancers, and we hope that they will be available for studies in LVAD patients. Specifically, AMG 386, a novel small peptide that blocks both Ang-1 and Ang-2, appears to reduce blood flow to tumors[46] and appears to be well tolerated by patients.[47] Similarly, the monoclonal antibody PF-4856884 reduces circulating levels of Ang-2 and reduces tumor blood flow.[48] Numerous other agents are currently in development.[4] The freshly isolated endothelial cells obtained from the patients in this study are vena caval in origin, although it is the capillary endothelial cells that likely contribute to deregulated angiogenesis in response to Ang-2. However, according to our hypothesis, both the stimulus (thrombin activity) and the effector (Ang-2) are circulating freely and are able to interact with all vascular beds. These systemic changes induce effects on vascular beds distinct from their origin, and systemic deregulation of the Ang/Tie-2 axis is a likely driver of angiogenesis. The use of warfarin and antiplatelets is an unavoidable confounder because nearly all LVAD patients use these drugs and the international normalized ratio goal in most non-LVAD patients taking warfarin is typically different from that of LVAD patients. Furthermore, common indications for warfarin in non-LVAD patients include atrial fibrillation, which itself is associated with increased Ang-2.[49] Although it is not possible to account for all confounding variables in human studies, we sought to minimize confounding through the enrollment of well-matched control groups. Although patients with OHT are useful to control for the effects of increased flow, sternotomy, and general anesthesia, patients with OHT are also treated with immunosuppressants, which are not used in patients with LVADs and may affect angiogenic signaling pathways.[50] Although this confounder is unavoidable, we have attempted to address this issue by enrolling 2 control groups, patients with OHT and patients with stable HF. Finally, whereas this series of experiments was designed to identify the molecular basis for deregulated angiogenesis in patients with LVADs, we acknowledge that blood flow conditions in patients with LVADs are markedly different from those in patients without LVADs. Although evidence suggesting a link between flow conditions and Ang-2 expression is limited and conflicting, we have chosen to focus on the molecular causes of Ang-2–dependent angiogenesis in this study. Although elevated Ang-2 was associated with a higher risk of NSB, the relationship between Ang-2 and AVM formation was not directly addressed in this study. Nevertheless, all of the patients who experienced NSB were found to have AVMs on endoscopy. However, patients who did not experience bleeding did not undergo endoscopy; therefore, the prevalence of asymptomatic AVMs in our study remains unknown. Despite these shortcomings, several studies have shown a strong association between Ang-2 and the development of vascular malformations,[9] small bowel angiodysplasia,[10] and increased capillary density.[51] Lastly, as a result of limitations in sample availability, we were unable to perform all assays in this study on all samples collected and therefore used subsets when necessary as indicated.

Conclusions

We have demonstrated that LVAD implantation is associated with increased thrombin-dependent overexpression of Ang-2, which leads to increased angiogenesis. Furthermore, we have shown that elevated Ang-2 is associated with NSB events in patients with LVADs. As the reliance on LVADs for the treatment of advanced HF continues to rise, the identification of novel therapeutic targets to treat LVAD-related complications will grow in importance. It remains to be determined whether pharmaceutical agents that antagonize Ang-2, which are currently in development, will hold promise for the treatment or prevention of LVAD-related AVM formation. Further studies are necessary to determine whether modulation of the Ang/Tie-2 pathway could have therapeutic benefits in reducing the complications associated with LVADs.

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DISCLOSURES

None.

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FOOTNOTES

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Elevated Angiopoietin-2 Level in Patients With Continuous-Flow Left Ventricular Assist Devices Leads to Altered Angiogenesis and Is Associated With Higher Nonsurgical Bleeding

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Table S1a – Clinical Characteristics of Participants in Figure 1 and 3

	HF	LVAD	OHT	*p*-value
Number of participants	17	38	14	
Age (years)	59.8±11.9	57.7±10.5	56.3±10.8	0.665
Female (%)	24	32	14	0.435
Black race (%)	41	47	29	0.281
Left Ventricular Ejection Fraction (%)	30.0±9.8	---	57.5±6.7	<0.001
Days Post Implant (Median)	---	295±483.5	253±805.4	0.609
BMI (kg/m2)	35.3±17.7	31.5±7.8	28.6±5.2	0.240
eGFR (ml/min*1.73m^2)	76.34±20.3	60.0±24.4	60.4±20.5	0.044
Dilated Cardiomyopathy (%)	53	63	36	0.206
Ischemic Cardiomyopathy (%)	41	42	43	0.995
Myocarditis (%)	0	2	14	0.113
Hypertension (%)	65	45	43	0.339
Diabetes mellitus (%)	6	43	64	0.003
Dyslipidemia (%)	41	55	64	0.419
NYHA HF Class (%)				<0.001
1	6	7	---	
2	19	70	---	
3	75	23	---	
4	0	0	---	
Heart rate (beats/min)	79.3±21.3	82.7±16.8	96.8±9.3	0.013
Mean Arterial Pressure (mmHg)	86.8±13.2	84.8±17.9	98.1±10.6	0.029
Pulse Pressure (mmHg)	41.4±13.3	29.6±8.5	47.4±9.4	<0.001
Hemoglobin (mg/dL)	12.9±1.5	11.4±1.7	12.5±2.0	0.009
B-type natriuretic peptide (ng/L)	2505.5±2245.6	2267.3±1851.6	---	0.693
Total cholesterol (mg/dL)	161.7±47.3	140.6±48.8	168.6±30.2	0.115
HDL (mg/dL)	50.1±24.1	35.7±14.4	42.8±14.3	0.023
LDL (mg/dL)	83.9±37.1	77.7±36.6	96.8±29.0	0.323

INR	1.6±0.7	1.9±0.5	1.1±0.1	<0.001
Platelet count (#/μL)	239.0±73.0	227.8±74.0	179.7±86.7	0.077
Statin (%)	53	61	100	0.011
Warfarin (%)	34	95	7	<0.001
ACE-I/ARB (%)	84	45	14	<0.001
Anti-platelets (%)	59	32	50	0.001

Table S1b – LVAD Parameters and Relevant Laboratories of Participants in Figures 1 and 3

	Heartmate II	HVAD	*p*-value
Number of participants	27	11	
Speed (rpm)	9061.2±374.7	2769.1±116.7	---
Flow (L/min)	5.4±1.3	4.9±1.1	0.291
Pulsatility Index	5.6±1.2	---	---
C-reactive protein (mg/dL)	67.8±68.5	38.5±43.3	0.516
Lactate dehydrogenase (U/L)	392.7±221.5	252.7±63.7	0.048

Table S2a – Clinical Characteristics of Participants in Figure 2

	HF	LVAD	OHT	*p*-value
Number of participants	10	13	12	
Age (years)	62.9±11.9	58.9±9.8	59.1±6.3	0.544
Female (%)	30	31	17	0.676
Black race (%)	50	31	25	0.499
Left Ventricular Ejection Fraction (%)	21.7±10.5	---	57.3±7.2	<0.001
Days Post Implant (Median)	---	166.0±662.7	324.0±396.2	0.367
BMI (kg/m2)	33.3±18.4	31.1±10.0	27.7±4.9	0.556
eGFR (ml/min*1.73m^2)	70.8±20.0	56.3±23.6	56.3±13.0	0.155
Dilated Cardiomyopathy (%)	50	77	42	0.177
Ischemic Cardiomyopathy (%)	50	15	50	0.123
Myocarditis (%)	0	8	17	0.376
Hypertension (%)	70	31	33	0.121
Diabetes mellitus (%)	10	39	67	0.026
Dyslipidemia (%)	50	69	67	0.604
NYHA HF Class (%)				<0.001
1	0	8	---	
2	11	54	---	
3	89	15	---	
4	0	0	---	
Heart rate (beats/min)	80.1±15.2	86.8±14.8	95.8±9.4	0.031
Mean Arterial Pressure (mmHg)	89.3±22.2	83.5±18.8	101.5±6.1	0.036
Pulse Pressure (mmHg)	42.1±14.7	31.2±12.1	47.8±9.9	0.006
Hemoglobin (mg/dL)	12.1±1.8	11.7±1.9	12.3±1.4	0.662
B-type natriuretic peptide (ng/L)	3282.4±2333.8	2114.1±1615.4	---	0.190
Total cholesterol (mg/dL)	160.3±66.3	146.7±50.8	172.0±31.0	0.553
HDL (mg/dL)	56.9±29.3	32.3±14.2	42.7±15.8	0.075
LDL (mg/dL)	81.0±45.4	78.6±34.4	99.4±30.7	0.430

INR	1.7±0.7	1.8±0.4	1.1±0.1	0.002
Platelet count (#/μL)	230.6±80.6	217.2±70.1	173.4±89.9	0.221
Statin (%)	50	54	100	0.015
Warfarin (%)	60	100	8	<0.001
ACE-I/ARB (%)	70	46	8	0.011
Anti-platelets (%)	50	92	50	0.039

Table S2b – LVAD Parameters and Relevant Laboratories of Participants in Figure 2

	Heartmate II	**HVAD**	***p*-value**
Number of participants	9	4	
Speed (rpm)	9042.2±343.5	2725.0±137.0	---
Flow (L/min)	5.0±0.6	5.1±1.9	0.965
Pulsatility Index	5.4±0.8	---	---
Lactate dehydrogenase (U/L)	418.2±150.7	257.8±58.7	0.068

Table S3a – Clinical Characteristics of Participants in Figures 4 and 5

	HF	LVAD	OHT	*p*-value
Number of participants	14	13	15	
Age (years)	65.4±11.8	58.0±11.1	50.2±12.7	0.009
Female (%)	35	23	27	0.697
Black race (%)	21	31	27	0.868
Left Ventricular Ejection Fraction (%)	30.5±7.4	---	61.7±9.3	<0.001
Days Post Implant (Median)	---	299.0±325.1	556.0±1575.5	0.046
BMI (kg/m2)	28.7±6.8	30.7±6.5	27.7±5.2	0.461
eGFR (ml/min*1.73m^2)	60.9±17.3	53.3±18.0	75.2±24.6	0.028
Dilated Cardiomyopathy (%)	50	54	40	0.902
Ischemic Cardiomyopathy (%)	36	39	33	0.902
Myocarditis (%)	0	8	7	0.590
Hypertension (%)	43	46	53	0.663
Diabetes mellitus (%)	36	31	27	0.891
Dyslipidemia (%)	57	46	40	0.663
NYHA HF Class (%)				0.001
1	0	15	---	
2	29	39	---	
3	64	31	---	
4	0	0	---	
Heart rate (beats/min)	75.9±13.6	88.6±12.7	93.3±12.8	0.006
Mean Arterial Pressure (mmHg)	86.8±10.2	75.3±19.4	93.3±12.8	0.012
Pulse Pressure (mmHg)	59.7±11.5	30.5±8.8	45.8±9.8	<0.001
Hemoglobin (mg/dL)	12.8±1.7	11.8±1.9	13.4±1.6	0.063
B-type natriuretic peptide (ng/L)	3039.8±4871.3	3837.7±2537.5	---	0.627
Total cholesterol (mg/dL)	190.6±61.0	122.3±50.1	163.5±40.4	0.028
HDL (mg/dL)	53.4±27.0	28.3±13.0	48.7±14.2	0.025
LDL (mg/dL)	112.1±50.0	64.9±41.5	90.3±30.1	0.066

INR	1.8±0.7	1.7±0.4	1.1±0.1	0.037
Platelet count (#/μL)	212.2±50.4	206.7±54.6	213.3±46.2	0.938
Statin (%)	36	69	93	0.014
Warfarin (%)	43	92	0	<0.001
ACE-I/ARB (%)	79	39	13	0.002
Anti-platelets (%)	36	85	53	0.054

Table S3b – LVAD Parameters and Relevant Laboratories of Participants in Figures 4 and 5

	Heartmate II	**HVAD**	***p*-value**
Number of participants	8	5	
Speed (rpm)	9222.0±628.4	2756.0±179.1	---
Flow (L/min)	6.1±1.2	4.3±1.5	0.044
Pulsatility Index	4.6±1.0	---	---
C-reactive protein (mg/dL)	80.0±16.4	27.0±21.2	0.049
Lactate dehydrogenase (U/L)	308.6±43.3	401.2±306.1	0.537

Table S4 – Biomarkers stratified by LVAD type

	HeartMate II	HVAD	*p*-value
Number of Participants	27	11	
VEGF (pg/mL)	149.93±202.70	140.39±140.26	0.525
Ang-2 (ng/mL)	9.45±7.92	19.36±9.96	0.002
Ang-1 (ng/mL)	3.73±3.39	3.73±4.44	0.751
Tie-2 (ng/mL)	21.25±5.99	26.36±7.72	0.101
Thrombin (RQ)	6.70±8.58	3.92±2.56	0.503

Table S5 – Biomarkers stratified by length of LVAD support

	Days of Support < Median	Days of Support > Median	*p*-value
Number of Participants	19	19	
VEGF (pg/mL)	148.16±170.04	146.18±204.62	0.885
Ang-2 (ng/mL)	13.31±8.23	11.32±10.89	0.181
Ang-1 (ng/mL)	4.31±4.21	3.15±3.02	0.452
Tie-2 (ng/mL)	22.49±5.71	22.97±7.972	0.863

Table S6a – Biomarkers in non-warfarin users

	Heart Failure	LVAD	Transplant	*p*-value
Number of Participants	11	2	13	
VEGF (pg/mL)	201.96±212.51	121.14±129.79	45.97±29.73	0.263
Ang-2 (ng/mL)	5.41±3.37	10.27±0.56	4.55±1.99	0.144
Ang-1 (ng/mL)	8.17±7.70	1.62±1.69	1.97±2.08	0.036
Tie-2 (ng/mL)	18.03±4.38	27.86±9.71	15.99±5.12	0.112

Table S6b – Biomarkers in warfarin users

	Heart Failure	LVAD	Transplant	*p*-value
Number of Participants	6	36	1	
VEGF (pg/mL)	148.11±128.11	148.62±189.43	109.12	0.689
Ang-2 (ng/mL)	8.26±3.50	12.43±9.83	2.28	0.227
Ang-1 (ng/mL)	3.95±3.79	3.84±3.72	6.13	0.524
Tie-2 (ng/mL)	19.43±4.20	22.45±6.73	16.91	0.477

Table S6c – Biomarkers in non-antiplatelet users

	Heart Failure	LVAD	Transplant	*p*-value
Number of Participants	7	3	7	
VEGF (pg/mL)	182.80±204.56	332.76±308.94	52.72±35.96	0.344
Ang-2 (ng/mL)	5.86±4.14	8.68±7.14	4.95±2.22	0.669
Ang-1 (ng/mL)	5.77±4.63	3.29±4.23	1.44±1.03	0.301
Tie-2 (ng/mL)	17.69±3.78	20.64±3.69	18.11±4.96	0.519

Table S6d – Biomarkers in antiplatelet users

	Heart Failure	LVAD	Transplant	*p*-value
Number of Participants	10	35	7	
VEGF (pg/mL)	180.97±176.17	131.27±169.01	48.25±32.86	0.212
Ang-2 (ng/mL)	6.81±3.33	12.63±9.77	3.83±1.74	0.007
Ang-1 (ng/mL)	7.26±7.86	3.77±3.68	3.02±2.90	0.485
Tie-2 (ng/mL)	19.20±4.70	22.91±7.06	13.99±4.25	0.007

Table S7a – Biomarkers stratified by aortic valve opening

	Never	Intermittently	Always	*p*-value
Number of Participants	18	9	11	
VEGF (pg/mL)	67.21±80.28	131.67±208.72	290.70±217.31	0.006
Ang-2 (ng/mL)	12.61±9.27	17.11±12.21	7.92±5.64	0.118
Ang-1 (ng/mL)	2.81±3.17	3.54±3.39	5.39±4.33	0.237
Tie-2 (ng/mL)	22.75±6.75	23.94±7.30	21.71±7.13	0.846

Table S7b – Non-Surgical Bleeding stratified by aortic valve opening

	Never	Intermittently	Always
Number of Participants	20	11	11
- Bleeding	90%	82%	91%
+ Bleeding	10%	18%	9%