THE UNIVERSITY OF CHICAGO

MOLECULAR MECHANISMS OF ANGIODYSPLASIA AND NON-SURGICAL BLEEDING IN HUMAN PATIENTS WITH CONTINUOUS-FLOW LEFT VENTRICULAR ASSIST DEVICES

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Chapter 1

Background and Introduction

Heart Failure (HF), a serious medical condition where the heart is unable to pump enough blood to meet the body's needs. HF is a major public health problem and is the most common cause of hospitalization in US adults[1]. Previously, long-term therapies available to treat heart failure included only medications or heart transplantation. In recent years, highly specialized implantable heart pumps known as left ventricular assist devices (LVADs) have emerged as durable tools to support the failing heart as a bridge to heart transplant or as a destination therapy. While the use of LVADs has significantly improved survival and quality of life in this patient population, non-surgical bleeding (NSB) – bleeding at a site distant from the operative site – commonly complicates the post-implantation course and frequently leads to morbidity and mortality[2].

LVAD-Related Non-Surgical Bleeding

NSB affects up to 30% of patients with LVADs and limits their more widespread use.[3] Angiodysplastic arteriovenous malformations (AVMs) in the gastrointestinal tract, nasopharynx, brain, and other tissues, are by far the most common cause of NSB in patients with LVADs[4]. This angiodysplasia represents new vessel growth within the effected tissue (usually the GI tract). It has been hypothesized that LVAD implantation may be directly or indirectly responsible for angiodysplasia and AVM formation as previous observational studies have

shown that LVAD removal normalizes the propensity to develop AVMs. However, the molecular mechanisms leading to angiodysplastic AVM formation and NSB in patients with LVADs are not known. This question however is of critical importance as NSB remains a significant cause of rehospitalization, increased healthcare cost, morbidity, procedural complications, and other poor outcomes in these patients. If the mechanism linking LVADs with angiodysplasia and NSB were known, novel medical therapies or changes to LVAD design could be explored to prevent or reduce these complications and improve patient outcomes.

Numerous studies have reported patient factors associated with NSB. These include older age, hypertension, operative time, low hematocrit, ischemic heart disease, high BMI, low albumin, female sex, and history of GI bleeding.[2, 5, 6] Importantly, other studies have linked renal dysfunction in LVAD patients with bleeding events,[7] and many angiogenic and inflammatory mediators are renally cleared. Interestingly, treatment with an angiotensin converting enzyme inhibitor (ACE-I) appears to be associated with reduced risk of bleeding in LVAD patients.[8] However, while these studies report important associations, they do not propose a pathophysiologic mechanism.

LVADs and von Willebrand Factor

Other reports have demonstrated platelet dysfunction,[9, 10] fibrinolysis,[11] and acquired von Willebrand syndrome[12] in LVAD patients suggesting that interaction between the LVAD device and the blood can cause deleterious effects and damage to blood components such as cells and clotting factors. Von Willebrand Factor (vWF) is a multimeric protein important in the coagulation cascade. Under basal conditions, approximately 50% of newly synthesized vWF is secreted immediately[13] while the remainder is stored in 1-6μm cigar-shaped organelles called

Weibel-Palade Bodies (WPBs) from which vWF can be secreted in response to a variety of physical and chemical stimuli[14]. vWF polymerizes in blood to form high-molecular weight multimers (HMWMs) which are cleaved by ADAMTS13 at a tightly controlled rate. Interestingly, up to 100% of patients with LVADs develop a deficiency in circulating vWF HMWMs - termed acquired von Willebrand Disease (AVWD) - that impairs the coagulation system[12, 15]. AVWD is associated with bleeding in 58% of patients[12] and the bleeding tendency correlates negatively with LVAD Pulsatility Index (PI)[16]. AVWD is also present in other forms of mechanical circulatory support (MCS) such as CentriMag support[17] and is reversed with removal of the LVAD[18]. While the exact mechanism leading to this deficiency is not known, multiple studies have suggested that mechanical shearing forces may stretch and deform the vWF HMWMs as they pass through the LVAD which may expose ADAMTS13 cleavage sites[19, 20]. The deformed vWF is rapidly degraded by ADAMTS13 leading to a significant deficiency in circulating vWF HMWMs.

A similar phenomenon was described in patients with Aortic Stenosis (AS) by Heyde[21]. In AS, disruption of vWF HMWMs correlates with severity of AS[22]. In patients with LVADs, bleeding correlates with PI[16]. In an *in vitro* flow circuit, disruption of vWF HMWMs correlates with duration of LVAD use[19]. However, it is not clear whether the vWF HMWMs display abnormalities prior to secretion in patients with LVADs. This is an important question as endothelial cells in these patients clearly display other forms of dysfunction, such as impaired nitric oxide synthesis. A strong association between continuous flow LVAD support and endothelial dysfunction has been demonstrated[23] and previous studies have shown that the transition from pulsatile flow to continuous flow may predispose patients with LVADs to bleeding. Additionally, the effect of AVWD on WPB dynamics and homeostasis is not known.

LVADs and Angiodysplasia

While platelet dysfunction and loss of vWF HMWMs may explain the coagulopathy in LVAD patients, these factors do not explain the angiodysplasia observed in up to 30% of LVAD patients. Despite numerous observational studies which link LVAD-related bleeding with various clinical characteristics, the molecular pathophysiology of LVAD-related angiodysplasia and bleeding is largely unexplored. To explain the angiodysplasia seen in LVAD patients, some authors have hypothesized that LVAD implantation can drive altered angiogenesis. In one study, myocardial capillary density was shown to increase after LVAD implantation[24]. In that study, capillary density was assessed microscopically in myocardial samples taken at the time of LVAD implantation and at the time of explantation in patients who either died or received a heart transplant. A significant increase in density was observed at the time of explant which could be one possible explanation for myocardial recovery after LVAD. This finding coupled with the known propensity for LVAD patients to develop angiodysplasia suggests that LVAD implantation may be associated with altered angiogenesis in multiple tissue types. This deregulation of angiogenesis may in fact be beneficial or deleterious to the patient's wellbeing depending on its location and the clinical setting. Therefore, understanding the physiological and/or pathophysiological mechanisms behind these abnormalities is imperative as such knowledge may lead to improvements in LVAD design, clinical practice, or therapeutics.

Molecular Drivers of Angiodysplasia

Angiogenic growth factors regulate the development of blood vessels. Altered growth

factor expression is associated with angiodysplasia in humans and animal models.[25] While vWF is the primary constituent within the WPBs, several vascular growth factors collocate with vWF within these organelles and are secreted from the endothelial cells in a tightly regulated fashion[14]. One notable molecule stored within WPBs is Angiopoietin-2 (Ang-2). The angiopoietins (Ang-1 and Ang-2) are a family of molecules that promote angiogenesis. Ang-2 is a Tie-2 antagonist synthesized by endothelial cells[26] in response to activation of the thrombin receptor.[27, 28] While Ang-2 exerts most of its effect on Tie-2 in an autocrine fashion, it does act in a paracrine and endocrine fashion as well. Ang-2 is potently angiogenic and strongly associated with altered vessel growth and angiodysplasia.[29-33] Ang-2 over-expressing mice develop redundant, tortuous, leaky capillaries and lesions in the alimentary tract[29] reminiscent of those in patients with LVADs.

Histologically, angiodysplasia is associated with increased endothelial proliferation in the setting of decreased coverage of the newly formed vessels by pericytes,[34, 35] non-endothelial vascular cells that support the endothelium and maintain endothelial quiescence. Pericytes produce Angoipoietin-1 (Ang-1), a Tie-2 agonist,[36] which promotes vessel stability and maturity.[37] As Ang-1 and Ang-2 exert opposing and complimentary actions on Tie-2 and ultimately on the endothelial cell itself, maintenance of proper Ang-1 and Ang-2 levels is critical for endothelial homeostasis. Derangement of this balance can lead to pathology. For example, a low Ang-1/Ang-2 ratio is associated with vascular malformations,[31] gastrointestinal angiodysplasia,[32] and increased capillary density.[33] While both Ang-1 and Ang-2 act in concert with VEGF to promote vessel growth, Ang-2 promotes endothelial activation, vascular destabilization, and inflammation[38].

Due to their potent effects on the endothelium, circulating levels of Angiopoietins are

very tightly regulated in the normal state. Ang-2 expression and release is potently induced by activation of the thrombin receptor (Protease-activated Receptor-1, PAR-1) on the endothelial cell surface[28, 39-44] and prior studies have suggested that plasma levels of thrombin may be elevated in patients with LVADs.[45-47] Ang-2 elevation has also been reported in patients with acutely decompensated heart failure[48] and has been shown to be a negative prognostic marker in HF patients at the time of hospital discharge[48]. Further, very high levels of Ang-2 have been reported in patients with severe shock[49]. Consistently, Ang-2 elevation is associated with negative outcomes. Additionally, a low Ang-1/Ang-2 ratio is associated with vascular disruption and may cause deregulation of angiogenesis in context-dependent concert with VEGF. Therefore, blockade of Ang-2 signaling is currently being investigated for use in the treatment of various cancers where neoangiogenesis is associated with accelerated tumor growth. Numerous Ang-2 blockers are commercially available and many have shown promise in decreasing cancer progression through angiogenic inhibition.[30] However, no study has investigated the balance of Ang-1 and Ang-2 after LVAD or the effect of Ang-2 blockade in the treatment of LVAD-related AVMs.

The complex mechanism by which Ang-2, and other WPB components (such as P-selectin, IL-8, Tie-2, and vWF) are secreted and regulated has been only partially described. Prior studies in cultured endothelial cells have shown that Ang-2 is secreted from endothelial cells on the surface of exosomes in a pathway regulated by the PI3K/Akt1 system[43]. However, overexpression of Ang-2 by adenovirus transfection results in a substantial free fraction of Ang-2 in culture medium as well as an increase in exosomal Ang-2[43]. Additionally, pharmacologic stimulators of vWF release such as phorbol myristate acetate (PMA) ester have been shown to induce Ang-2 release in cultured human umbilical vein endothelial cells (HUVECs) beginning at

~5 minutes and peaking at ~20 minutes of stimulation[50], possibly through a protein kinase C (PKC)-dependent mechanism. This rate of exocytosis closely mirrors that of vWF under identical conditions. Removal of PMA from the culture medium results in detectable intracellular Ang-2 recovery within 6 hours and return of the storage granules within 16 hours[50]. Pharmacologic inhibition of Ang-2 has been shown to inhibit angiogenesis and decrease tumor growth in mice[51].

Other WPB components may also contribute to bleeding and vascular inflammation in LVAD patients. For example, P-selectin acts to increase adhesion of platelets and leukocytes to the endothelium when the endothelial cell is activated and IL-8 induces systemic inflammation and leukocyte migration. Upon activation of the endothelial cell by histamine, thrombin, tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), or phorbol esters, P-selectin becomes surface-expressed[52]. vWF-deficient mice have decreased P-selectin expression on the surface of endothelial cells[53] and mice deficient in P-selectin have been shown to have increased bleeding time[54]. Further, overexpression of Ang-2 has been shown to obliterate P-selectin expression[50] suggesting a mechanistic link between the synthesis, storage, and kinetics of P-selectin and Ang-2. Ang-2 and P-selectin have been shown to be stored in mutually exclusive WPBs and sometimes even in completely different endothelial cells[50]. While it is not known which populations of WPBs respond to various stimuli and whether stimulant-induced secretion occurs via exosomes, these findings demonstrate a critical link among angiogenic, thrombotic, and inflammatory processes.

Tumor Necrosis Factor-α (TNF-α) is a key inflammatory mediator that is critically linked with the Angiopoietin system. TNF-α promotes abnormal angiogenesis in synergy with Ang-2[55] and regulates Ang-2 expression in cell culture models.[56] TNF-α also induces apoptosis of

pericytes which produce Ang-1.[57-59] Further, TNF-α induces vascular leakage[60] and promotes surface expression of Tissue Factor (TF, Factor III) in endothelial cells.[61] TF produces thrombin in the plasma which in turn increases expression and release of Ang-2 from the endothelium. These effects are augmented in synergy with thrombin itself.[62, 63] Prior studies have suggested that inflammation may be elevated in patients with LVADs.[64] In this way, Ang-2 and TNF-α are intimately linked and may act together to drive angiodysplasia and vascular instability in LVAD patients. However, the mechanism by which TNF-α contributes to vascular destabilization in LVAD patients has not yet been described.

However, vascular destabilization typically involves endothelial cell contraction and migration, as well as increased endothelial permeability. Rho-associated Coiled-coil Kinase (ROCK) is a downstream effector of RhoA, a small GTPase, and regulator of actin cytoskeletal organization. Among other functions, ROCK regulates cellular contraction and motility and its activity is upregulated by inflammatory mediators. Specifically, TNF-α activates ROCK in endothelial cells,[65] which leads to endothelial contraction and permeability under inflammatory or thrombotic conditions or after vascular injury. ROCK also regulates endothelial cell migration in response to vascular endothelial growth factor (VEGF)[66] and endothelial cell organization during angiogenesis.[67] Therefore, if Ang-2 and TNF-α act together to regulate LVAD-related angiodysplasia and vascular destabilization, it is highly likely that ROCK acts as a downstream affecter of both and that inhibition of ROCK could prevent or treat LVAD-related NSB.

While coordinated activation of thrombin, TF, TNF-α, Ang-2, and ROCK could explain a molecular mechanism for LVAD-related NSB, these factors do not explain why some patients bleed and other do not. Only 30% of LVAD patients experience NSB while the remaining 70%

never experience a bleeding event. If Ang-2 and TNF-α together drive LVAD-related angiodysplasia, and this angiodysplasia leads to bleeding, it remains unclear why some patients may experience deregulation of these factors with subsequent bleeding and others never experience bleeding at all. Further, the overwhelming predilection of LVAD-related NSB for the GI tract remains unexplained.

The Microbiome and Angiodysplasia in LVAD Patients

Differences in the gastrointestinal microbiome may explain why some LVAD patients bleed and others do not. Butyrate is a 4-carbon short chain fatty acid (SCFA) produced through fermentation of dietary fiber by specific gut microbes including *Eubacterium rectale/Roseburia spp.* and *Faecalibacterium prausnitzii*.[68, 69] Butyrate strongly inhibits the expression of both TNF-α[70] and Ang-2[71] and blunts TNF-α/Ang-2-dependent vascular destabilization.[29, 72-75] Oral butyrate supplements are widely available and butyrate treatment reduces gut inflammation and angiogenesis in humans and animal models.[76-81] However, LVAD patients systematically restrict dietary fiber consumption as fiber-containing foods frequently also contain high levels of Vitamin K. LVAD patients must avoid consumption of Vitamin K which counters the anticoagulant effect of warfarin, a pharmaceutical agent universally used to prevent LVAD thrombosis. Further, LVAD patients are commonly treated with antibiotics to prevent frequent driveline infections. Together, these dietary fiber restrictions and antibiotic treatment may cause loss of butyrate-producing microbes (dysbiosis) and lower circulating butyrate levels which lead to vasculopathy.[82] Taken together, loss of butyrate-producing microbes in LVAD patients may lead to the over-expression of TNF-α and Ang-2 and the development of angiodysplasia in the gut. However, the interplay among the microbiome, TNF-α, and Ang-2 has not been explored in

relation to LVAD-related NSB.

Research Approach in this Project

To date, the effects of LVAD implantation on the vasculature (specifically the endothelium) remain poorly studied due in large part to a lack of appropriate small animal models of chronic mechanical circulatory support. Simply put, a modern LVAD is approximately the size of a human fist and therefore too large to fit into a small rodent model. For this reason, research in this area is restricted to the use of a bovine model, which is costly and impractical for large-scale molecular studies, or samples obtained from humans with LVADs which are difficult to obtain in large scale.

To remedy these problems, I developed several novel methods in the course of this study. To date, few laboratories have reliably isolated and studied endothelial cells from living human subjects without the use of surgical arterectomy, a process which is impractical for use in living humans at serial time points. Using a minimally invasive endothelial biopsy from living human subjects[83, 84], I investigated whether derangement of growth factor expression in the vasculature may lead to increased angiogenesis in patients with LVADs which may in turn function as the basis for angiodysplastic AVM formation. I also developed new methods for measuring the net physiological effects of markers in the blood from patients with LVADs by incubating cultured human vascular cells in dishes with plasma or serum from patients with or without LVADs. In this way, I was able to quantify the net physiologic effect of alterations in circulating levels of these markers on the benchtop. While these novel techniques carry with them numerous limitations, they have allowed me to describe for the first time a linear molecular mechanism which could explain the cause of LVAD-related angiodysplasia and bleeding, a critical first step

to exploiting these pathways for clinical benefit, or blocking them to reduce complications.

Chapter 2

Thrombin-induced Angiopoietin-2 Overexpression in Human Patients with Continuous-Flow Left Ventricular Assist Devices Induces Abnormal Blood Vessel Growth and Predicts Non-Surgical Bleeding Events

As reported in

Tabit CE, Chen P, Kim GH, Fedson SE, Sayer G, Coplan MJ, Jeevanandam V, Uriel N, Liao JK. *Elevated Angiopoietin-2 Level in Patients with Continuous-Flow Left Ventricular Assist Devices Leads to Altered Angiogenesis and Is Associated with Higher Non-Surgical Bleeding.* Circulation. 2016 Jul 12;134(2):141-52.

Abstract

Background: Non-surgical bleeding (NSB) is the most common adverse event in patients with continuous-flow left ventricular assist devices (LVADs) and is caused by arteriovenous malformations (AVMs). We hypothesized that deregulation of an angiogenic factor, Angiopoietin-2 (Ang-2), in LVAD patients leads to increased angiogenesis and higher NSB.

Methods: Ang-2 and thrombin levels were measured by ELISA and Western blotting, respectively, in blood samples from 101 patients with heart failure (HF), LVAD, or orthotopic heart transplant (OHT). Ang-2 expression in endothelial biopsy was quantified by immunofluorescence. Angiogenesis was determined by *in vitro* tube formation using serum from each patient with or without Ang-2-blocking antibody. Ang-2 gene expression was measured by RT-PCR in endothelial cells incubated with plasma from each patient with or without the thrombin receptor blocker Vorapaxar.

Results: Compared with HF or OHT patients, serum levels and endothelial expression of Ang-2 were higher in LVAD patients ($p=0.001$ and $p<0.001$, respectively). This corresponded with 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 NSB 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 NSB events. Ang-2 therefore may contribute to AVM formation and subsequent bleeding in LVAD patients.

Introduction

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 concert with vascular endothelial growth factor (VEGF)[30]. In contrast, Ang-2 is synthesized exclusively by endothelial cells and is stored with von Willebrand Factor in Weibel-Palade Bodies.[26] Upon exocytosis from endothelial cells, Ang-2 antagonistically binds to Tie-2, competitively inhibiting Ang-1 and promoting altered vessel growth in concert with VEGF.[30] In short, while both Ang-1 and Ang-2 act in concert with VEGF to promote angiogenesis, Ang-1 promotes normal vessel growth while Ang-2 promotes abnormal growth associated with vascular destabilization and inflammation.[30, 38]

Activation of the thrombin receptor (Protease-activated Receptor-1, PAR-1) on the endothelial cell surface promotes Ang-2 expression and release from endothelial cells.[28, 39, 41-43] Prior studies have suggested that plasma levels of thrombin may be elevated in patients with LVADs.[45-47] Given the known relationship between thrombin-dependent PAR-1 activation and Ang-2 expression and release, we hypothesized that thrombin-induced Ang-2 overexpression may promote altered blood vessel growth in patients with LVADs.

Methods

Study Subjects

A cross sectional study was performed. The study included 3 groups of patients, all treated at the University of Chicago Medical Center. Adult patients supported with an LVAD (Thoratec Heartmate II or Heartware HVAD) at least 30 days post-implantation served as the

experimental group. We employed two control groups: heart failure patients with reduced ejection fraction (HFrEF) (defined as a left ventricular ejection fraction less than 40%) without an LVAD, and patients with history of orthotopic heart transplantation (OHT) at least 30 days post transplantation. We chose to have two control groups in this study because neither control group is a perfect comparator to LVAD patients. 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 heart failure, active cancer within 1 year, untreated hypoxic conditions, acute thrombosis within 6 months, severe renal disease defined as an estimated glomerular filtration rate (eGFR) less than 30 ml/min*1.73m^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 using an automated blood pressure cuff which has recently been reported as the most accurate method for measuring blood pressure in patients with LVADs.[85] All subjects 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 and Passaging

Human umbilical vein endothelial cells were purchased from Lonza (Basel, Switzerland). Upon arrival, cells were rapidly thawed in a 37 ºC water bath. One cryovial of thawed cells was

then mixed with 20mL of Endothelial Growth Medium-2 (EGM-2, Lonza) and carefully pipetted into a T-75 flask (Falcon). The flask containing the cells and medium was then incubated under standard conditions (37C, 5% CO_2) overnight. In the morning, the cells were inspected for adhesion. The medium was aspirated under aseptic technique and replaced with 20mL of EGM-2. This medium was similarly changed every 48h. Once the culture reached 70% confluence, the cells were washed in PBS under gentle rocking. The PBS was aspirated and the cells were detached using 2mL of 0.025% Trypsin/EDTA (Lonza) for 5 minutes. Once detached, the Trypsin was neutralized using 2mL of Trypsin Neutralizing Solution (TNS, Lonza) and the cells were then mixed with 56mL of EGM-2. The mixture was then evenly divided among 3 T-75 flasks which were returned to the incubator. This process was repeated when the cells again reached 70% confluence. For all experiments, HUVECs were used for experiments prior to passage 7.

Measurement of Circulating Biomarkers

Peripheral venous blood was obtained from patients by antecubital venipuncture under aseptic technique using an 18-gauge butterfly needle and collected in vacutainer tubes (BD Bioscience) containing ethylenediaminetetraacetic acid (EDTA), sodium heparin, sodium citrate, or silica clot activator. Samples were placed on ice and quickly transported from the hospital back to the laboratory. Samples were immediately centrifuged at 2000 x *g* for 20 minutes at 4 ºC. The plasma and serum fractions were collected, divided into 1.5mL microcentrifuge tubes, and frozen at -80 ºC for future analysis. Because platelet activation causes the release of VEGF and Ang-1, levels of these markers were measured in platelet-poor plasma (EDTA) by ELISA

(R&D Systems, Minneapolis, MN). Levels of Ang-2 and soluble Tie-2 (sTie-2) levels were similarly measured in serum. Aliquots of samples were first diluted in buffer per each ELISA kit's manufacturer instructions. Standards were prepared by serial dilution. For each kit, other necessary buffers and reagents were reconstituted and/or diluted according to each manufacturer's instructions. Recommended volumes of diluted samples or standards were then carefully pipetted into each well of the kit's microplate in duplicate. The wells were sealed with adhesive film and the plates were incubated at RT with constant agitation for the recommended lengths of time. The wells were then individually aspirated, using caution not to scratch the bottom of each well. The wells were washed in buffer 3 times and aspirated. Conjugated antibody specific to the marker of interest was then added to each well. Again, the wells were sealed, incubated, agitated, and washed in triplicate. Finally, recommended volumes of substrate solution were pipetted into each well and the microplates were incubated at RT in the dark for the recommended times. Stop solution was then added to each well and the plates were then immediately read on a plate reader set to 450nm. The plates were then read again at 570nm and the values subtracted to correct for optical imperfections in the plate. Using the controls on each plate, a standard curve was developed. Concentrations of each analyte were then determined in each sample using this standard curve.

As Thrombin and Prothrombin cannot be readily differentiated by ELISA due to common antibody binding sites, levels were measured by Western Blot and differentiated by molecular weight. As certain phlebotomy techniques could induce artefactual thrombin formation, both thrombin and prothrombin were measured in plasma (EDTA) meticulously drawn through an 18-gauge butterfly needle from the antecubital vein. This plasma was immediately transported on ice to the laboratory and centrifuged at 2000g for 10 minutes before being aliquoted into 1.5mL

microcentrifuge tubes and frozen at -80 ºC within 30 minutes of phlebotomy. Thrombin and prothrombin levels were then determined by Western Blot using a Bio-Rad system as follows. Samples were thawed on ice, vortexed, and centrifuged at 10,000g and 4 ºC for 20 minutes. 10µL of sample was then mixed with 90µL of Sample Buffer (4% SDS, 20% Glycerol, 0.004% Bromophenol Blue, 0.125M Tris-HCl, and BME 10% in dH_2O), vortexed, and boiled at 95 ºC for 3 minutes before being cooled on ice. Polyacrylamide (8%) gels were prepared and 20uL of sample was loaded into each lane. Electrophoresis was then performed at 100V and 4 ºC. Gels were then carefully removed from the glass plates and transferred to a 0.45µm Immobilon® membrane at 60V and 4 ºC overnight. Membranes were then blocked in 3% BSA/PBST for 1 hour at 4 ºC. 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) were prepared in 3% BSA/PBST solution. Factor XIIa and Factor XIa levels were measured similarly 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). After blocking, membranes were incubated with primary antibody at 4 ºC for 12h under gentle rocking. Membranes were then washed in PBST and incubated with secondary antibody for 12h under gentle rocking. Membranes were then again washed in PBST in triplicate and laid flat on the stage of a Bio-Rad ChemiDoc imaging system with Bio-Rad Image Lab 5.1 software. Peroxide solution containing Luminol (Bio-Rad) was then carefully pipetted onto each membrane and protein expression was measured by densitometry. Each sample was assayed in triplicate and the results averaged.

Peripheral Endothelial Cell Collection

Vena caval endothelial cells were obtained from guidewires used during right heart catheterization using adaptation of several methods previously described.[83, 84, 86-88] Central venous access was obtained using a modified Seldinger technique and a 6F venous sheath was advanced into the common femoral vein or internal jugular vein over an 0.035 inch J-wire (Arrow International, Reading PA). The J-wire was advanced as far as possible through the inferior vena cava and the vessel wall was gently abraded. The wire was then retracted through the sheath taking care to open the diaphragm on the end of the sheath to prevent cell loss. The wire was then cut using wire cutters and placed into a 50mL conical tube containing 30mL of Dissociation Buffer (2.5g BSA, 0.05g heparin, and 2mL 0.5M EDTA, in 500mL PBS without Ca or Mg). The tube was gently inverted to coat the wires with the buffer and the tube was then immediately placed on ice and transported back to the laboratory. The tube was then gently vortexed to dissociate the cells from the wires. The wires were removed from the tube and discarded. The 50mL conical tube was then centrifuged at 400g for 7 minutes at 4 ºC. The supernatant was carefully aspirated and the pellet was resuspended in red blood cell lysis buffer for 10 minutes. After the red cells had lysed, the solution was diluted with wash buffer and again vortexed at 400g for 10 minutes at 4 ºC. Again, the supernatant was aspirated and the cell pellet was resuspended in PBS. Glass 4-well culture-slides (Fisher) were previously coated with poly-L-lysine for 30 minutes at 37 ºC. These were then aspirated and washed with PBS. The cells in PBS were then pipetted on these culture slides. The slides were then affixed in a centrifuge and spun at 400rpm briefly to facilitate adhesion of the cells to the poly-L-lysine-coated slides. The slides were then rotated 180º in their holders and spun again at 400rpm. The slides were then removed from the centrifuge and very gently aspirated. Ice cold 4% paraformaldehyde was

gently pipetted to each well and the slides were incubated on the benchtop for 10 minutes. The paraformaldehyde was then aspirated and the slides were washed twice in PBS. The slides were then dried in a fume hood to facilitate laminar flow of air over the slide tray. Once thoroughly dry, the slides were stored at -80 ºC until analysis.

Assessment of Endothelial Protein Expression by Quantitative Immunofluorescence

To measure the expression of Ang-2 in the endothelium of each patient, endothelial biopsy samples were analyzed by quantitative immunofluorescence using methods we have previously described.[83] Slides with adherent fixed endothelial cell samples were thawed to RT and rehydrated with 50mM glycine in PBS for 10 minutes. The cells were then permeabilized using 0.01% Triton X for 10 minutes and washed in glycine/PBS in triplicate. Primary antibodies were prepared in 0.5% BSA/50mM glycine in PBS against Ang-2 (1:150 dilution; R&D Systems, Minneapolis, MN) and vWF (1:300 dilution; Dako, Carpentaria, CA) followed by fluorescent-labeled secondary antibodies (1:200 dilution; Invitrogen, Carlsbad, CA). After blocking with 0.5% BSA for 10 minutes, the cells were incubated with primary antibody at 37C for 1 hour. The slides were then washed and incubated with secondary antibody for 45 minutes. The slides were then washed again and dried. Finally, the slides were mounted under glass coverslips with Vectashield containing DAPI for nuclear identification (Vector Laboratories, Burlingame, CA). For each batch of patient-derived cells, a control slide of cultured HUVECs taken from a single passage were stained contemporaneously. Slides were imaged on an Olympus BX41 fluorescent microscope at 20x magnification and analyzed using Image J software.[89] Endothelial cells were manually. Fluorescent intensity of Ang-2 was quantified in

20 cells from each patient and 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. All quantifications were performed by a technician blinded to patient identity and cohort.

Assessment of the Effect of Angiopoietin-2 on Angiogenesis

To assess the angiogenic effect of Ang-2 in each patient's blood on angiogenesis, we incubated cultured HUVECs with serum from each patient. First, 24-well cell culture plates (Falcon) were coated with Matrigel (Corning Life Sciences, Corning, NY) which was allowed to solidify at 37C 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 (EBM-2, Lonza) with growth factor additives such that the final concentration of each exogenous growth factor in the serum/EBM-2 mixture was equal to that in EGM-2 (Lonza). This mixture containing 200,000 HUVECs was then gently pipetted into the Matrigel-coated wells and incubated for 18h under standard conditions in the presence or absence of an Ang-2 blocking antibody 150ng/mL (azide-free mouse-anti-human-Ang-2, Adipogen, San Diego, CA) 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 (8μg/mL, Corning) to improve microtube visibility and microtube formation was assessed by microscopy [90]. Total number of mircotubes in a low power field were quantified

visually (5 fields per well were averaged). All quantifications were performed by a technician blinded to patient identity and cohort. Each patient sample was assayed in triplicate and the results averaged.

Assessment of the Effect of Thrombin on Angiopoietin-2 Expression

To assess the effect of thrombin in patients' blood on Ang-2 expression, we incubated cultured HUVECs with plasma from each patient, anticoagulated with fondaparinux 1mcg/mL, and measured Ang-2 gene expression by RT-PCR. Fondaparinux, a Factor Xa inhibitor, was used for several reasons. First, the commonly used anticoagulants sodium citrate and EDTA are cytotoxic at the doses required to achieve full anticoagulation and therefore cannot be used with cultured endothelial cells. The remaining commonly used anticoagulant sodium heparin promotes binding of thrombin to antithrombin which inhibits thrombin activity, making heparin a poor choice for an assay intended to measure thrombin activity. Fondaparinux provided the added benefit of preventing further conversion of prothrombin to thrombin by Factor Xa while the plasma samples incubate with the HUVECs. As vacutainer tubes containing fondaparinux are not commercially available, we added 50mcg fondaparinux to 50mL conical tubes. Blood was obtained from an antecubital vein through an 18-gauge butterfly needle and collected in a 50mL syringe. We then dispensed the blood from the 50mL syringe into the tube immediately upon collection. Samples were gently mixed manually, centrifuged at 2000G for 20 minutes at 4 ºC, aliquoted, and frozen at -80 ºC. HUVECs grown to 70% confluence on 6-well plates (Falcon) under standard conditions were starved overnight in EBM-2 supplemented with 2% fetal bovine serum (FBS, Life Technologies) but devoid of supplemental growth factors. To

ensure adequate thrombin receptor blockade, cultures were then incubated with the thrombin receptor blocker Vorapaxar 100µg/mL (Adooq Bioscience, Irvine, CA) or vehicle for 2 hours. During this time, the frozen plasma samples obtained previously were warmed to 37 ºC and centrifuged at 2000g for 10 minutes. The plasma sample from each patient was then divided and mixed with either Vorapaxar 100µg/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 4 hours under standard conditions. After incubation, the plasma was aspirated and the cultures were washed with PBS. The HUVECs were immediately lysed and harvested by manual scraping. RNA was isolated by column prep using a PureLink RNA Mini Kit (Life Technologies).

Ang-2 gene expression was then measured by RT-PCR. First, concentration of harvested RNA was determined photometrically. Equal amounts of RNA were then aliquoted and the harvested RNA was reverse transcribed to cDNA using a Bio-Rad reverse transcription kit. The qPCR reaction plates were loaded with dNTPs, reverse transcriptase, SYBR green, water, and cDNA template according to the manufacturer's instructions. GAPDH served as a loading control. qPCR was then performed and Ang-2 expression was determined relative to measured GAPDH expression.

Statistical Analyses

Statistical analyses were preformed using SPSS version 23.0. Continuous variables such as biomarkers were compared among groups using the Kruskal-Wallis test, followed by pairwise *post hoc* comparisons using 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 using the Wilcoxon Signed-Rank test. Categorical variables such as bleeding events were compared using Fisher's exact test. Pearson correlation was used to evaluate the relationship between serum levels of Ang-2 and endothelial tube formation on Matrigel. Clinical characteristics were compared using ANOVA or Student's t-test for continuous variables or Pearson Chi-Square testing for categorical variables as appropriate. Ordinal variables such as NYHA HF class were compared using the Wilcoxon Rank Sum test. Data are presented as mean ± standard deviation unless otherwise indicated. A 2-sided *P* value of <0.05 was considered statistically significant.

Results

We enrolled 32 patients with HF, 44 patients with LVADs, and 25 patients with OHT. OHT patients were included to control for the effects of sternotomy, general anesthesia, and increased cardiac output. Clinical characteristics are shown in Table 2.1 with subgroups shown in the subsequent tables. All groups were similar in age, sex, race, and renal function. As expected, the NYHA HF Class was higher (worse) for the HF group compared with the LVAD group.

Both the LVAD and OHT groups were studied approximately 300 days post-implant. Among the LVAD cohort, 32 patients with a Thoratec Heartmate II and 12 patients with a Heartware HVAD were studied. LVAD flow (HM2 5.4±1.2, HVAD 4.6±1.3 L/min, p=0.077), C-reactive protein (HM2 77.9±44.8, HVAD 39.2±37.5 mg/dL, p=0.192), and lactate dehydrogenase (HM2

Table 2.1: Clinical Characteristics

	HF	LVAD	OHT	*p*-value
Number of participants	32	44	25	
Age (years)	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
Days Post Implant (Median)	---	295.0±479.2	311.0±1257.9	0.100
BMI (kg/m2)	32.7±13.9	30.7±7.6	28.1±4.7	0.217
eGFR (ml/min*1.73m^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 (beats/min)	78.1±17.5	83.0±16.9	95.1±11.8	0.001
Mean Arterial Pressure (mmHg)	88.3±14.7	84.4±17.6	96.2±11.1	0.011
Pulse Pressure (mmHg)	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 (#/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
Anti-platelets (%)	53	90	52	<0.001

386.7±206.0, HVAD 309.3±205.0 U/L, p=0.272) were similar between both groups (Table 2.2). Pulse pressure was slightly higher in the HVAD group compared with Heartmate II (35.17±12.50 vs. 27.86±5.64 mmHg, 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. On average, both the LVAD and OHT groups were studied approximately 300 days post-implant.

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

To evaluate the circulating levels of Ang-2, Ang-1, sTie-2, and VEGF in patients with and without an LVAD, we measured serum levels of Ang-2 and sTie-2 and platelet-poor plasma levels of Ang-1 and VEGF by ELISA (Figure 2.1a-d). Notably, Ang-2 was higher in patients with LVADs compared 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 vs. LVAD p=0.012, LVAD vs. OHT p=0.003). Soluble Tie-2 was similarly elevated in patients with LVADs (LVAD 22.73±6.85, HF 18.97±4.39, and OHT 16.05±4.92 ng/mL, omnibus p=0.004, HF vs. LVAD p=0.213, LVAD vs. OHT p=0.004), possibly due to 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, 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 compared with HF[91] but is decreased after OHT[92]. 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

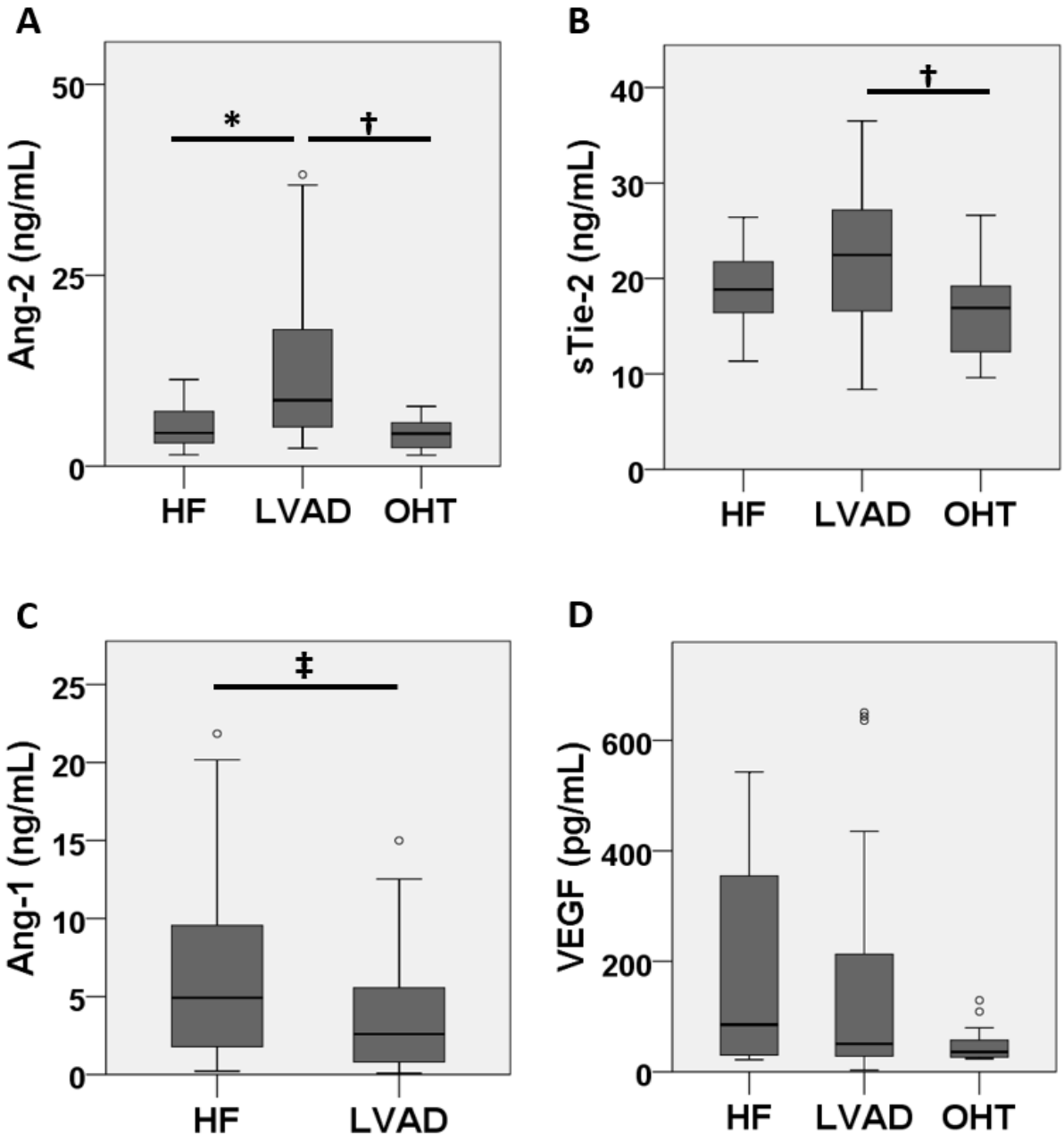


Figure 2.1: Altered blood levels of angiogenic proteins in patients with LVADs. *Blood levels of Ang-1, Ang-2, VEGF, and Tie-2 were measured by ELISA and patients with HF, LVAD, or OHT (n=17, 38, and 14 respectively). (a) Ang-2 was significantly higher in LVAD patients compared with HF and OHT. (b) Soluble Tie-2 (sTie-2) was significantly higher in LVAD patients compared with OHT and increased non-significantly from patients with HF. (c) Ang-1 trended lower in LVAD patients compared with HF. (d) Plasma VEGF remained elevated in patients with LVADs compared with HF but trended lower in patients with OHT. * $p<0.05$, † $p<0.01$, ‡ $p=0.055$*

Table 2.2 – LVAD Parameters and Relevant Laboratories

	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 2.3 – 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 2.4– 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

presence of continued over-expression of VEGF, a constellation that favors abnormal angiogenesis.[93-96] In subset analysis, Ang-2 was significantly higher in LVAD patients with HVAD vs. Heartmate II without a significant difference in the other biomarkers measured (Table 2.3). No significant relationship was noted between length of LVAD support and any biomarker

Table 2.5 – 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 2.6 – 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 2.7 – 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

tested (Table 2.4). The relationships between biomarkers and warfarin or antiplatelets were challenging to interpret due to extremely small patient numbers in the sub-groups (Tables 2.5 to 2.8). Levels of VEGF appeared to be higher in LVAD patients whose aortic valve opened with every beat while no relationship was seen between aortic valve opening and the other biomarkers (Table 2.9).

Table 2.8 – 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 2.9 – 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

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 HF or OHT (52.4±19.9, 24.2±10.2, 35.0±11.2 AU, omnibus $p<0.001$, HF vs. LVAD $p<0.001$, LVAD vs. OHT $p=0.029$) (Figure 2.2). These findings suggest that over-expression of Ang-2 in the endothelium may be responsible for the elevated circulating Ang-2 levels in patients with LVADs.

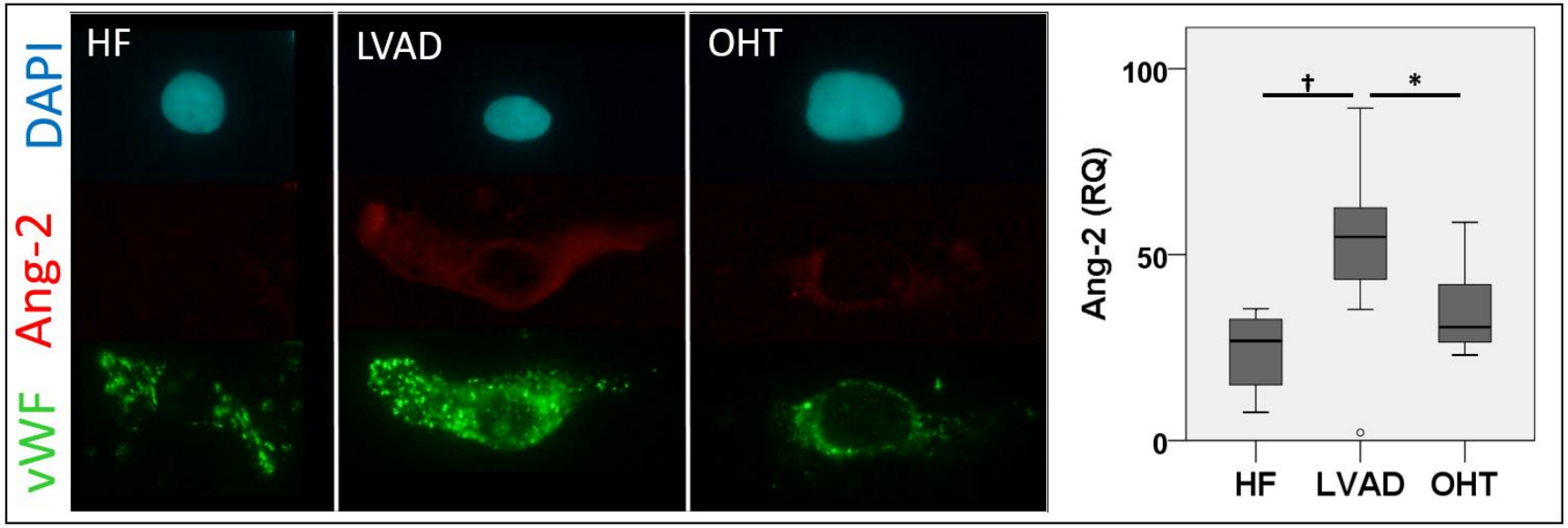


Figure 2.2: Endothelial Angiopoietin-2 expression is increased in patients with LVADs. *Endothelial biopsy was performed in patients with HF, LVAD, or OHT (n=10, 13, and 12 respectively) and endothelial cells were isolated by centrifugation. Cells were plated onto microscope slides and stained with fluorescent-labeled antibodies. Protein content was measured by quantitative immunofluorescence. Endothelial expression of Angiopoietin-2 (red) was significantly higher in patients with LVADs compared with HF or OHT. * $p<0.05$, † $p<0.01$*

Elevated Ang-2 in Serum from Patients with LVADs Induces Angiogenesis

Previous studies have shown that Ang-2 increases endothelial tube formation on Matrigel.[97] To investigate whether 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 vs. LVAD $p=0.003$, LVAD vs. OHT $p<0.001$) (Figure 2.3). This effect was abolished by the Ang-2 blocking antibody (20.71±6.98 tubes per low power field respectively, $p<0.001$), indicating that elevated Ang-2 levels 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 groups 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 2.4).

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.[45-47] 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

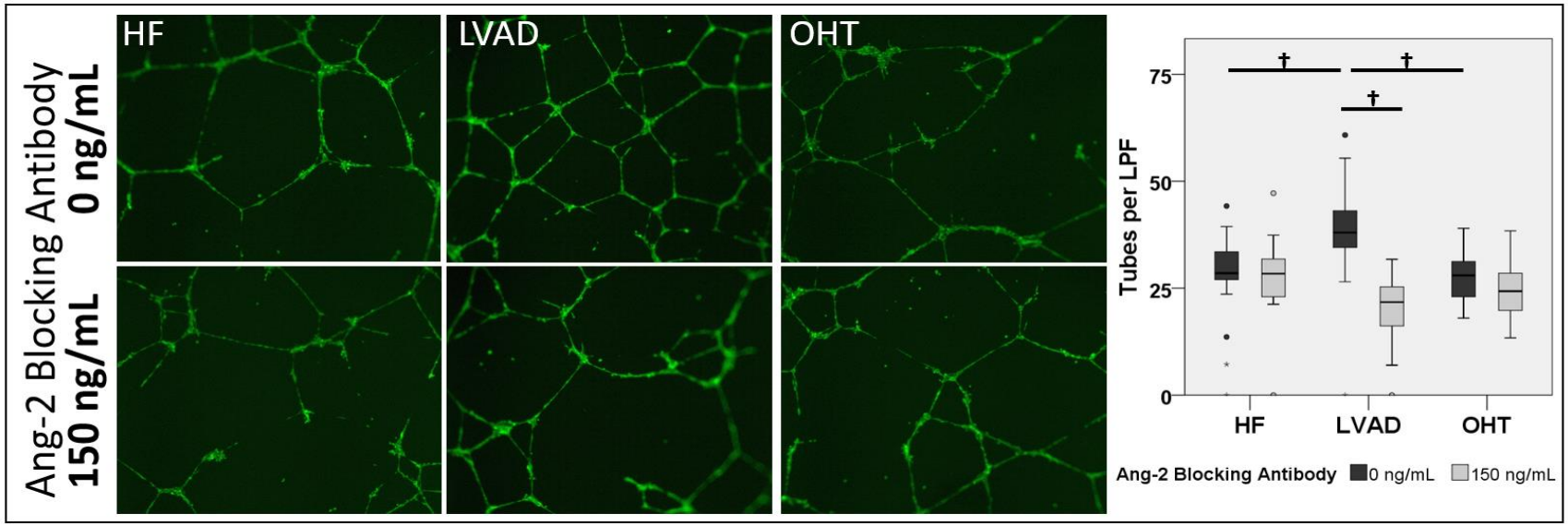


Figure 2.3: Elevated Angiopoietin-2 in serum from patients with LVADs induces angiogenesis in human endothelium. *Human umbilical vein endothelial cells were assayed on Matrigel and incubated overnight with serum from patients with HF, LVAD, or OHT (n=17, 35, and 14 respectively) in the presence or absence 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 controls. Co-treatment with 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. † $p<0.01$*

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 vs. LVAD p=0.039, LVAD vs. OHT p=0.003) (Figure 2.5a-b). Prothrombin, however, was not different among the three 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 Western Blot. Both Factor XIIa (HF 0.50±0.26, LVAD 1.01±0.58, and OHT 0.63±0.28 AU, omnibus p=0.037, HF vs. LVAD p=0.034, LVAD vs OHT p=0.377) and Factor XIa (HF 0.94±0.47, LVAD 2.29±1.49, OHT 0.86±0.21 AU, omnibus p=0.014, HF vs. LVAD p=0.060, LVAD vs. OHT p=0.028) were higher in LVAD patients (Figure 2.5c-f), suggesting that activation of the

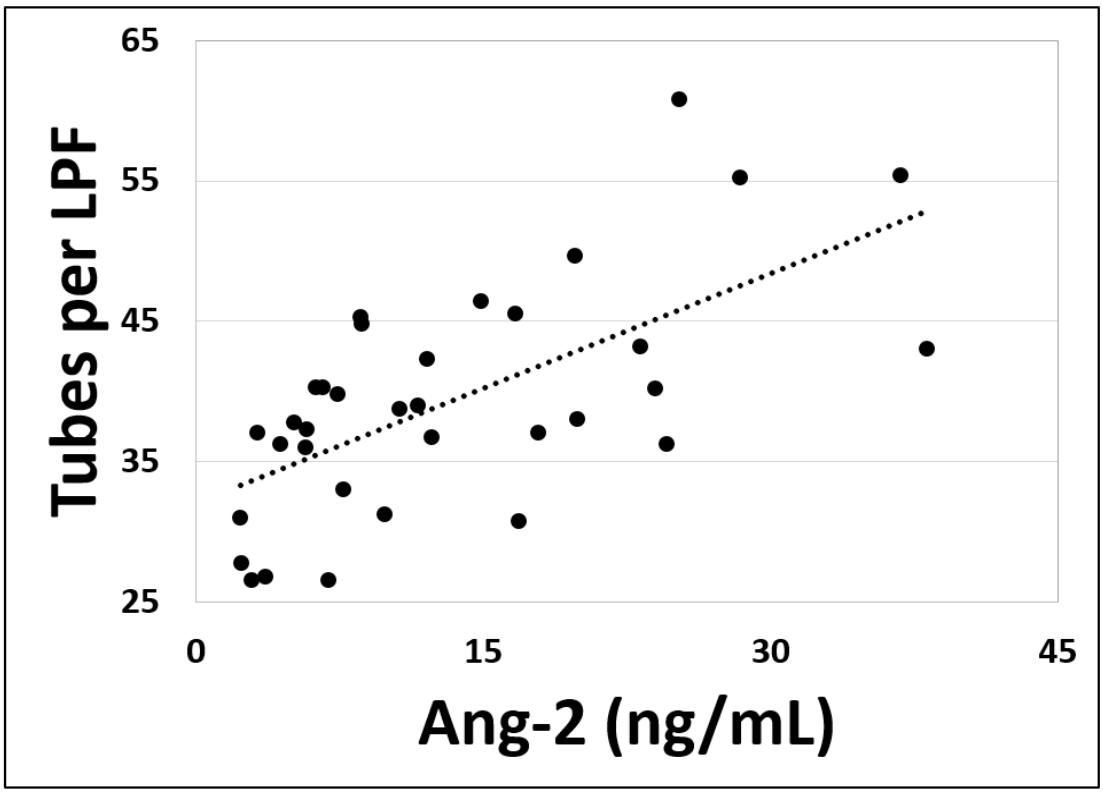


Figure 2.4: Among LVAD patients, tubule formation correlates strongly with serum Ang-2 concentration. *R^2=0.645, p<0.001*

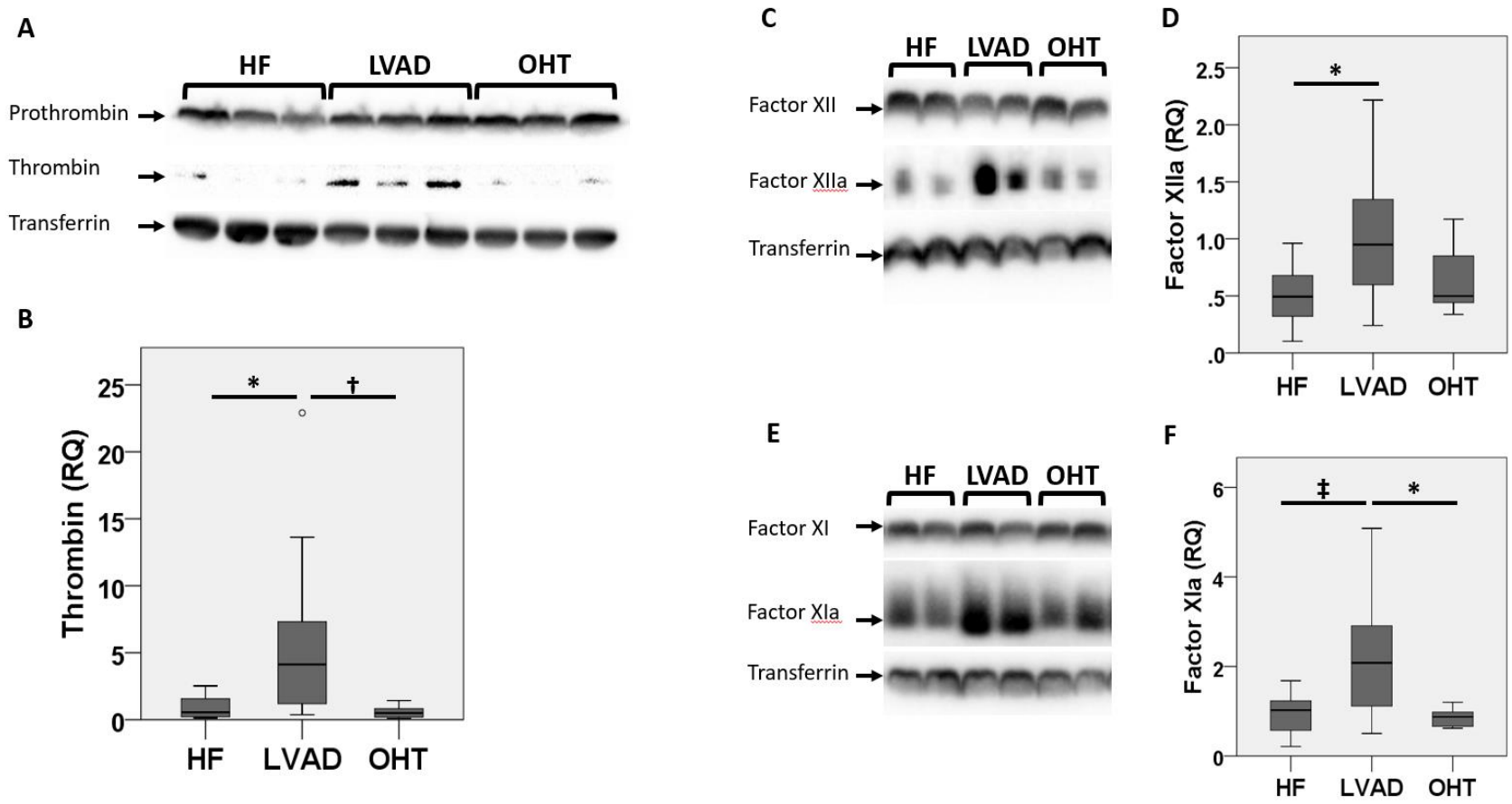


Figure 2.5: ***Elevated plasma levels of thrombin in patients with LVADs.*** *Thrombin was measured by Western Blot in plasma from patients with HF, LVAD, or OHT (n=14, 13, and 15 respectively). (a-b) Thrombin was significantly higher in plasma from patients with LVADs compared with HF or OHT while prothrombin was unchanged. To investigate one possible source of this elevated thrombin, we measured Factor XIIa and XIa by Western Blot in plasma from these patients. (c-f) Both Factor 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. * $p<0.05$, † $p<0.01$, ‡ $p=0.060$*

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 RQ respectively, omnibus $p<0.001$, HF vs. LVAD

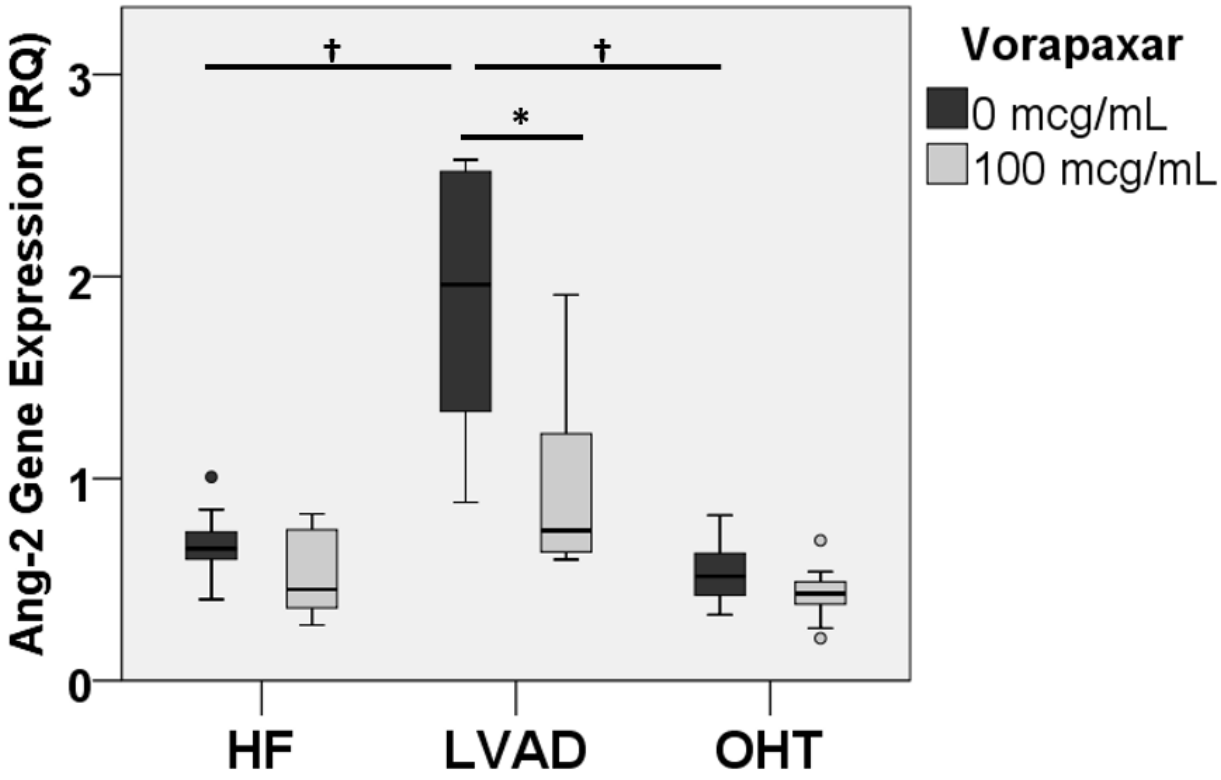


Figure 2.6: Elevated plasma levels of thrombin in patients with LVADs induce endothelial overexpression of Ang-2. *Plasma samples from patients with HF, LVAD, or OHT (n=14, 13, and 15 respectively) were anticoagulated with fondaparinux to prevent artefactual thrombin generation ex vivo. Cultured HUVECs 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 RT-PCR. Plasma from patients with LVADs induced significantly higher expression of Ang-2 compared with HF or OHT. This effect was normalized with thrombin receptor blockade. * $p<0.05$, † $p<0.01$*

p=0.003, LVAD vs. OHT p<0.001) (Figure 2.6). This increased Ang-2 expression was significantly reduced with thrombin receptor blockade (0.95±0.22 RQ, p=0.013). In contrast, a small, but non-significant 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 RQ respectively, p=NS). Taken together, these data suggest that elevated thrombin in plasma from patients with LVADs induces increased endothelial Ang-2 expression.

Elevated Ang-2 in Serum from Patients with LVADs is Associated with an Increased 3-month Risk of GI Bleeding Events

To investigate the role of serum Ang-2 in predicting NSB events in patients with LVADs, we reviewed the electronic medical record of all patients with an LVAD enrolled in the present study for instances of GI bleeding, intracranial hemorrhage (ICH), or epistaxis. GI bleeding was defined as a report of blood in the stool or vomitus, a finding of hemoccult positive stool, or a finding of pathologic 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 pathologic bleeding within the nasopharynx on endoscopy. Among patients with an LVAD and a serum Ang-2 level above the mean of 12.32 ng/mL (n=13), 5 patients had at least one NSB event within 3 months of sample collection (4 GI bleeds, 1 epistaxis, 0 ICH) while 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 GI bleeds, 1 epistaxis, 0 ICH), suggesting a critical time interval between 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, $p<0.001$).

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 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 inter-cellular connections that are associated with vessel maturation[75] and induces endothelial inflammation and abnormal angiogenesis,[93-96] resulting in tortuous, fragile vessels that are prone to bleeding. Indeed, Ang-2 over-expressing mice develop dilated, tortuous, redundant vessels[29] reminiscent of AVMs in patients with LVADs. Blockade of Ang-2 signaling is currently being investigated in the treatment of various cancers where neovascularization is associated with accelerated tumor growth.[30] Numerous Ang-2 blockers are commercially available and many have shown promise in decreasing tumor size[30]. However, it is not known whether Ang-2 blockade can prevent or is an effective therapy for LVAD-related NSB.

In the current 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, and 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 implant,[98] 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*[28] and prior studies have suggested thrombin activity may be increased in patients with LVADs[45, 46] due to interaction of coagulation factors with the materials of the LVAD.[47] Specifically, our data suggest that the contact coagulation system (regulated by Factor XIIa and Factor XIa) appears to be activated in LVAD patients, which could be one 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[99] suggesting that the use of alternate materials or inhibition of the contact coagulation system might improve LVAD hemocompatibility and/or reduce complications.

In addition to thrombin's role in converting fibrinogen to fibrin, thrombin is known to activate PAR-1.[40, 41] PAR-1 activates the Phospholipase-C-β signaling cascade, inducing Weibel-

Palade Body exocytosis and Ang-2 expression.[43, 44] 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 (Figure 2.7). 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 as all patients in our study with an HVAD received the device as part of a bridge-to-transplant (BTT) strategy while patients with a Heartmate II used as BTT 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.[45, 47] While one limited study did suggest the presence of increased thrombin activity in modern LVAD recipients (through observation of fibrin split products and thrombin/antithrombin complexes),[46] an increase in circulating active thrombin after LVAD has not been shown previously 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[100] and patients with LVADs retain a significant risk of LVAD thrombosis despite the use of warfarin.[101] 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 demonstrating 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.[102] Wever-Pinzon and colleagues

reported an association between pulsatility and NSB in LVAD recipients.[16] However, in the present study, we found no significant association between aortic valve opening or pulse pressure and NSB events or Ang-2 expression. While 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 Ang-2 expression is increased by continuous flow versus pulsatile flow[103] while others suggest the opposite.[104] Furthermore, while 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 due to high shear forces produced by the LVAD.[105, 106] Also, flow in the small arterioles, capillaries, and venous system is normally non-pulsatile 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 non-pulsatile. Therefore, a non-mechanical cause of Ang-2 overexpression is more consistent with known data as 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 pharmacologic 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, which blocks both Ang-1 and Ang-2, appears to reduce blood flow to tumors[107] and appears well tolerated by patients.[108] Similarly, the monoclonal antibody PF-4856884 reduces circulating levels of Ang-2 and reduces tumor blood flow.[109] Numerous other agents are currently in development.[30] The freshly isolated endothelial cells obtained from the patients in this study are vena caval in origin while 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 are unavoidable confounders as nearly all LVAD patients use these drugs and the INR goal in most non-LVAD patients taking warfarin is typically different from that of LVAD patients. Further, common indications for warfarin in non-LVAD patients include atrial fibrillation which itself is associated with increased Ang-2.[110] While 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. While patients with OHT are useful to control for 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.[111] While this confounder is unavoidable, we have attempted to address this issue by enrolling two control groups, patients with OHT and patients with stable HF. Finally, while 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 patients without LVADs. While 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. While 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, and 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,[31] small bowel angiodysplasia,[32] and increased capillary density.[33] Lastly, due to 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.

In summary, we have demonstrated that LVAD implantation is associated with increased thrombin-dependent overexpression of Ang-2, which leads to increased angiogenesis.

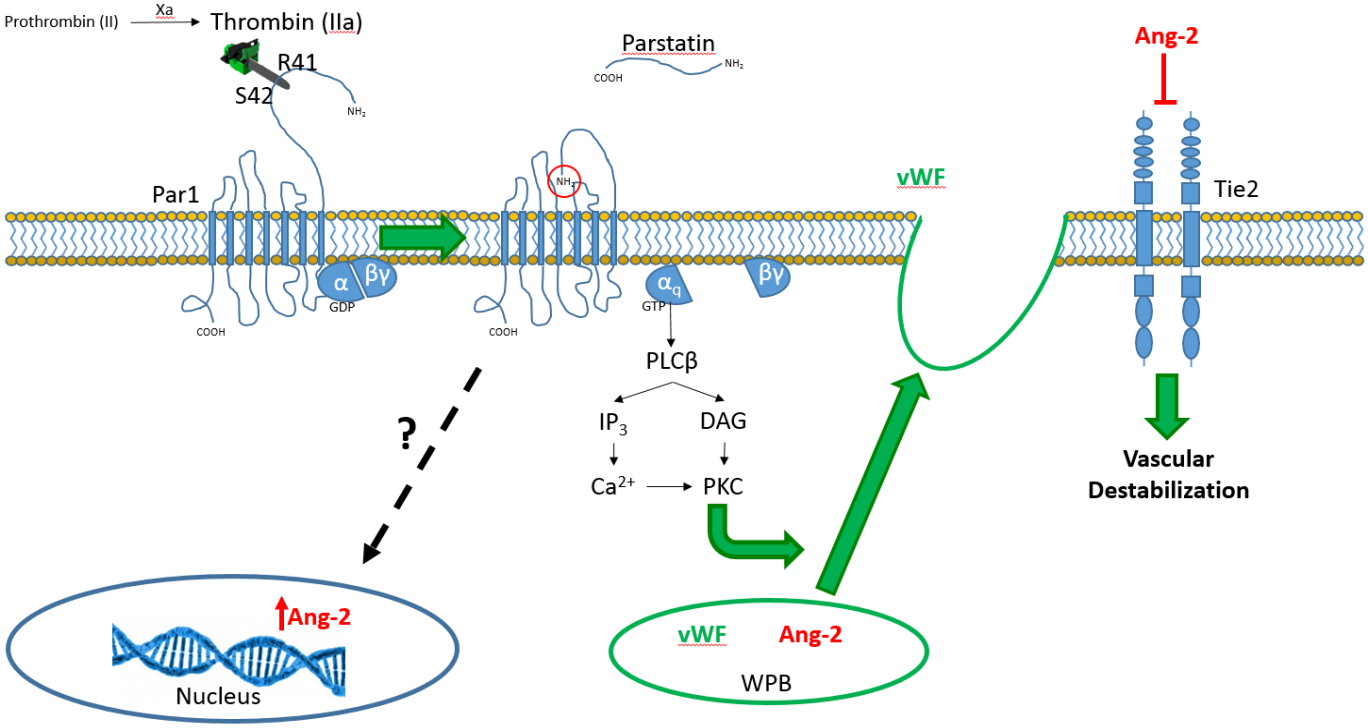


Figure 2.7: Thrombin-induced Par1 activation may explain the increase in Ang-2 expression and angiogenesis associated with LVAD implantation. *Prothrombin is cleaved by Factor Xa to form active Thrombin. Thrombin formation is increased in patients with LVADs due to interaction of coagulation factors with the materials of the LVAD. Thrombin is known to cleave the inhibitory tail of Par1, allowing the new N-terminus to bind and activate the receptor. The cleaved tail (Parstatin) dissociates into the blood. Par1 then activates the PLCβ signaling cascade which induces Ang-2 release through previously described mechanisms. Ang-2 then binds to and antagonizes Tie-2 resulting in vascular destabilization and increased angiogenesis in concert with VEGF. Factor Xa (Xa), Protease-activated Receptor 1 (Par1), Phoshpolipase Cβ (PLCβ), Inositoltriphosphate (IP_3), Diacylglycerol (DAG), Intracellular Calcium (Ca^{2+}), Protein Kinase C (PKC), von Willebrand Factor (vWF), Weibel-Palade Body (WPB), Angiopoietin-2 (Ang-2), TEK Tyrosine Kinase Receptor (Tie-2).*

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 heart failure 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.

Chapter 3

Increased Tumor Necrosis Factor-α Levels in Patients with Continuous-Flow Left Ventricular Assist Devices Mediate Vascular Instability and Are Associated with Higher Non-Surgical Bleeding

As reported in

Tabit CE, Coplan MJ, Chen P, Jeevanandam V, Uriel N, Liao JK. *Increased Tumor Necrosis Factor-α Levels in Continuous-Flow Left Ventricular Assist Devices.* Journal of Heart and Lung Transplantation. 2017 Jun 8. S1053-2498(17)31834.

Abstract

Background: Non-surgical bleeding (NSB) due to angiodysplasia is common in patients with LVADs. Previously, we reported that thrombin-induced Angiopoietin-2 (Ang-2) expression in patients with LVADs leads to altered angiogenesis and is associated with decreased Angiopoietin-1 (Ang-1) and increased NSB. However, the mechanism for decreased Ang-1, which is made by pericytes, is not known. Furthermore, the etiology of thrombin activation in patients with LVADs is still not clear. This study aims to assess if high levels of Tumor

Necrosis Factor-α (TNF-α) in patients with LVADs induce pericyte apoptosis, promote Tissue Factor (TF) expression and thrombin activation, and promote vascular instability.

Methods: TNF-α and TF levels were measured by ELISA in blood from 101 patients with heart failure (HF), LVAD, or orthotopic heart transplant (OHT). Cultured human pericytes were incubated with serum from each patient with or without TNF-α blockade. Ang-1 expression was measured by RT-PCR and pericyte death was measured by fluorescent live/dead stain. TF gene expression was measured by RT-PCR in cultured human endothelial cells incubated with plasma from each patient with or without TNF-α blockade. TF expression in endothelial biopsy samples from these patients was measured by quantitative immunofluorescence. Finally, cultured human endothelial cells were incubated on Matrigel with serum from each patient with or without TNF-α-blocking antibody and tube formation was assessed by microscopy.

Results: LVAD patients had higher plasma levels of TNF-α compared with HF or OHT patients. Serum from LVAD patients suppressed Ang-1 expression in pericytes more than serum from patients without LVADs. Serum from LVAD patients induced more pericyte cell death than serum from HF or OHT patients. Both plasma and endothelial cells from LVAD patients contained higher amounts of TF, and plasma from LVAD patients induced more TF expression in endothelial cells than plasma from HF or OHT patients. Further, serum from LVAD patients had more angiogenic potential than serum from HF or OHT patients. All of these effects of LVAD serum were reversed or reduced with TNF-α blockade. Interestingly, all NSB events in the LVAD cohort occurred in patients with high plasma levels of TNF-α.

Conclusions: Elevated TNF-α in LVAD patients is a central regulator of altered angiogenesis, pericyte apoptosis, TF expression, and thrombin activation. Our findings suggest that blockade of TNF-α may have therapeutic benefits in reducing vascular complications in LVAD patients.

Introduction

Continuous-Flow Left Ventricular Assist Devices (LVADs) are a mainstay therapy for advanced heart failure (HF). However, non-surgical bleeding (NSB) – bleeding at a non-operative site – complicates the post-LVAD course in up to 30% of patients.[3] Angiodysplasia of the gastrointestinal (GI) tract and nasopharynx is the most common cause of NSB in patients with LVADs.[4] However, the underlying mechanism remains poorly understood.

Angiodysplasia represents pathologic vessel growth and is associated with increased endothelial proliferation in the setting of decreased coverage of the newly formed vessels by pericytes,[34, 35] non-endothelial vascular cells that support the endothelium and maintain endothelial quiescence. Pericytes produce Angoipoietin-1 (Ang-1), an agonist of Tie-2,[36] which promotes vessel stability and maturity.[37] Ang-1 is antagonized by Angiopoietin-2 (Ang-2) which is synthesized by endothelial cells[26] in response to thrombin.[27, 28] Ang-2 induces endothelial destabilization, inflammation, and abnormal vessel growth.[30, 38] We have recently shown that plasma levels of Ang-1 are lower in LVAD patients compared with controls[112] although the mechanism underlying this decrease of Ang-1 in LVAD patients is not known. We have also shown that thrombin-dependent overexpression of Ang-2 in LVAD patients induces altered angiogenesis and is strongly associated with NSB events.[112] Since Ang-1 and Ang-2 are critical regulators of vascular growth and development, determining the mechanisms underlying the loss

of Ang-1 and overexpression of Ang-2 in LVAD patients is critical to the understanding of LVAD-associated angiodysplasia and NSB.

As mentioned above, pericytes are a main source of Ang-1 and Tumor Necrosis Factor-α (TNF-α) is a key pro-inflammatory mediator that induces pericyte apoptosis.[57-59] Further, TNF-α promotes abnormal angiogenesis in synergy with Ang-2[55, 113] and regulates Ang-2 expression in cell culture models.[56] Interestingly, TNF-α also induces the expression of Tissue Factor (TF, Factor III) in endothelial cells[61], which leads to thrombin production in the plasma. These effects are augmented in synergy with thrombin itself.[62, 63] Prior studies have suggested that inflammation may be elevated in patients with LVADs.[64] Therefore, TNF-α may play a central role in the promotion of angiodysplasia in LVAD patients through the dysregulation of Angiopoietins and TF. However, the activity of TNF-α and its interplay with angiogenic and coagulation pathways in LVAD patients has not been explored. We hypothesized that high levels of TNF-α in LVAD patients induce pericyte apoptosis, decrease Ang-1 expression, induce endothelial TF expression, and promote altered angiogenesis and vascular instability in the presence of high Ang-2 levels.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and grown using Endothelial Growth Medium-2 (EGM-2, Lonza) on T-75 flasks (Falcon). Similarly, human brain pericytes were purchased from ScienCell (Carlsbad, CA) and grown on T-75 flasks (Falcon) using Pericyte Medium (ScienCell). All cultures were grown

under standard conditions (37 ºC, 5% CO_2). Cells were grown to 70% confluence, washed in PBS, trypsinized, and passaged. Cultured cells were used for all experiments prior to passage 7.

Measurement of the Effect of TNF-α on Vascular Cells

To investigate the effect of TNF-α and thrombin on TF expression in endothelial cells, we incubated HUVECs with TNF-α (50ng/μL), Thrombin (1 unit/μL), TNF-α/Thrombin together, or control medium under standard conditions for 4 hours. Cells were then lysed, RNA was isolated using column preps, and TF gene expression was measured by RT-PCR.

To investigate the effect of TNF-α and Ang-2 on angiogenesis, we incubated HUVECs with TNF-α (10ng/mL) in DMEM with 10% FBS or control medium (DMEM with 10% FBS) for 48 hours. Cells were then rested overnight in DMEM with 10% FBS. Cells were then trypsinized and resuspended in EBM-2 with 20% FBS with or without Ang-2 (100ng/mL). This mixture was then pipetted into Matrigel (Corning) coated 24-well plates and incubated overnight under standard conditions. Tubule formation was then measured by microscopy.

To investigate the effect of TNF-α and Ang-2 on Ang-1 expression in pericytes, we incubated cultured pericytes with TNF-α (50ng/μL), Ang-2 (100ng/μL), TNF-α/Ang-2 together, or control medium for 4 hours under standard conditions. Cells were then lysed, RNA was isolated by column preps, and Ang-1 gene expression was measured by RT-PCR.

To investigate the effect of TNF-α and Ang-2 on pericyte apoptosis, we incubated cultured pericytes with TNF-α (50ng/μL), Ang-2 (100ng/μL), TNF-α/Ang-2 together, or control medium overnight under standard conditions. Cells were then lysed, protein was isolated, and

the ratio of Caspase-3/Procaspase was determined by western blot. We then incubated cultured pericytes with increasing doses of TNF-α (0, 1, 5, 10, or 100ng/μL) for 24 hours under standard conditions and measured apoptosis by flow cytometry using an Annexin-V/Propidium Iodide kit (Abcam). To investigate the synergic effect of TNF-α/Ang-2, we then incubated cultured pericytes with TNF-α (10ng/μL), Ang-2 (100ng/μL), TNF-α/Ang-2 together, or control medium for 24 hours under standard conditions and again measured apoptosis by flow cytometry.

Recruitment, Inclusions, and Exclusions of Participants

We performed a cross sectional study including 3 groups of patients: (1) Adult patients supported with an LVAD (Thoratec Heartmate II or Heartware HVAD), (2) heart failure patients with reduced ejection fraction (HFrEF, defined as a left ventricular ejection fraction less than 40%) without an LVAD, and (3) patients with a history of orthotopic heart transplantation (OHT). OHT patients were included to control for the effects of sternotomy, general anesthesia, and a massive increase in cardiac output, which occur in patients receiving an LVAD but not in the HF group without an LVAD. All LVAD/OHT patients were recruited more than 30 days after implantation to avoid confounding due to the acute effects of surgery. Patients were recruited in the cardiac catheterization laboratory or the outpatient Cardiology clinic and were in clinically stable condition at the time of enrollment. Patients with decompensated heart failure, cancer within 1 year, untreated hypoxia, acute thrombosis within 6 months, severe renal disease (eGFR less than 30 ml/min*1.73m^2), untreated autoimmune diseases, or acute illness of any kind were excluded. Clinical information was obtained from the electronic medical record. LVAD parameters were obtained from each patient's LVAD control module at the time of recruitment. All blood samples were obtained while patients were in the fasting state. The study protocol was

approved by the University of Chicago Institutional Review Board and all participants provided written informed consent.

Adult patients in stable condition were recruited from the Advanced Heart Failure Clinic at the University of Chicago or from the cardiac catheterization laboratory if the patient had previously been seen in the Advanced Heart Failure Clinic. Three groups of patients were studied: Patients supported with an LVAD (n=32 Thoratec Heartmate II and 12 Heartware HVAD) at least 30 days post-implantation, functionally similar heart failure patients with reduced ejection fraction (HFrEF, LVEF <40%) without an LVAD, and patients with a history of OHT at least 30 days post-transplantation. OHT patients were included to control for the effects of sternotomy, general anesthesia, and increased cardiac output. All patients were in clinically stable condition at the time of enrollment and sampling. Patients were excluded if they had decompensated heart failure, active cancer within 1 year, untreated hypoxic conditions, acute thrombosis within 6 months, severe renal disease, or acute illness of any kind. All subjects were studied in the fasting state. As shown in Table 1, the groups were largely similar except in areas where differences would be expected (such as LVEF between HF and OHT patients). Despite efforts to enroll functionally similar HF and LVAD patients, the HF patients without an LVAD in this study were still slightly sicker (as measured by NYHA functional class), which was expected and theoretically could bias our study toward the null as worsening HF is known to increase systemic inflammation[88, 114] and associated biomarkers.[115-117] Subgroup comparisons are shown in Tables S1-4.

Measurement of Circulating Biomarkers

Venous blood was collected in vacutainer tubes (BD Bioscience) containing ethylenediaminetetraacetic acid (EDTA) or silica clot activator. Samples were centrifuged immediately at 2000*g* for 20 minutes at 4 ºC. The plasma/serum fraction was collected, divided, and frozen at -80 ºC for future analysis. TNF-α and TF were measured in platelet-poor plasma by ELISA (TNF-α: Life Technologies; TF: R&D Systems). Serum was used for other experiments as described below.

Assessment of the Effect of TNF-α on Angiopoietin-1 Expression in Pericytes

Cultured pericytes were grown to 70% confluence on 6-well plates (Falcon) under standard conditions. Serum samples from patients with and without LVADs were diluted 1:1 with DMEM, mixed with TNF-α-blocking antibody (100ng/mL, Cell Signaling) or vehicle, and incubated at 37C for 2 hours with gentle intermittent mixing to allow neutralization of the TNF-α in the serum/DMEM mixture. For this assay, we did not include serum from patients with OHT because all OHT patients in our cohort were treated with tacrolimus at the time of the study, which inhibits Ang-1 expression.[111] After 2 hours, the cultures were aspirated and the serum/DMEM samples with or without TNF-α-blocking antibody were added to each well. Cultures were then incubated for an additional 4 hours under standard conditions. After incubation, the serum/DMEM was aspirated and the cultures were washed with PBS. RNA was isolated using a PureLink RNA Mini Kit (Life Technologies) and Ang-1 gene expression was measured by RT-PCR.

Assessment of the Effect of TNF-α on Pericyte Cell Death

Cultured pericytes (ScienCell) were grown to 70% confluence on 96-well plates (Falcon) under standard conditions. Serum samples from patients with LVAD, HF, or OHT were diluted 1:1 with DMEM, mixed with TNF-α-blocking antibody (100ng/mL, Cell Signaling) or vehicle, and incubated at 37C for 2 hours with gentle intermittent mixing to allow neutralization of the TNF-α in the serum/DMEM mixture. After 2 hours, the cultures were aspirated and the serum/DMEM samples with or without TNF-α-blocking antibody were added to each well. Cultures were then incubated for an additional 12 hours under standard conditions. After incubation, the serum/DMEM was aspirated and the cultures were washed with PBS. Cultures were stained with a fluorescent viability kit (Thermo Fisher Scientific) according to manufacturer instructions. Briefly, Calcein AM and Ethidium dimer were mixed at the recommended concentrations and pipetted into each well. Calcein labels live cells while Ethidium labels the nuclei of dead or dying cells. Intensity of each marker was then determined on a plate reader. To control for well-to-well variation in seeding density, pericyte death was measured as the ratio of dead to live cells in each well. Three wells for each sample and treatment condition were averaged.

Harvesting Endothelial Cells from Patients

We obtained vena caval endothelial cells from discarded guide wires used during right heart catheterization as previously described.[83, 84, 86-88, 112] Briefly, central venous access was obtained using a modified Seldinger technique and a venous sheath was placed into the femoral vein or internal jugular vein over a guide wire (Arrow International, Reading PA). The wire was

advanced through the vena cava and endothelial cells were collected by gentle abrasion with the vessel wall. Endothelial cells were recovered from the wire by centrifugation and plated onto poly-L-lysine coated microscope slides (Sigma, St. Louis, MO). Cells were fixed immediately in 4% paraformaldehyde, washed in PBS, dried, and stored at -80 ºC until further processing.

Assessment of Endothelial Tissue Factor Expression by Quantitative Immunofluorescence

Samples were analyzed as described previously.[83] Briefly, fixed endothelial cells were thawed, washed in PBS, and stained with primary antibodies against TF (1:300 dilution; Abcam) and von Willebrand Factor (vWF, 1:300 dilution; Dako, Carpentaria, CA), followed by fluorescent-labeled secondary antibodies (1:200 dilution; Invitrogen, Carlsbad, CA), and then mounted under glass coverslips with Vectashield mounting medium with DAPI to aid in nuclear identification (Vector Laboratories, Burlingame, CA). To control for batch-to-batch variability in staining, a control slide of cultured HUVECs taken from a single passage was stained contemporaneously with each batch of slides from patients. Slides were imaged on an Olympus BX41 fluorescent microscope at 100x magnification and analyzed using Image J software.[89] Fluorescent intensity of TF was measured in 20 randomly selected cells from each patient and the results averaged. Average fluorescent intensity for each patient sample was then normalized to the average intensity of the HUVEC control slide for the corresponding batch. 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. Analysis was performed by blinded technicians.

Assessment of the Effect of TNF-α in LVAD Patients on Endothelial Tissue Factor Expression

Plasma was obtained from an antecubital vein and anticoagulated with fondaparinux (1 μg/mL). Samples were mixed, centrifuged at 2000*g* for 20 minutes at 4 ºC, divided, and frozen at -80 ºC. Cultured HUVECs were grown to 70% confluence on 6-well plates (Falcon) under standard conditions and starved overnight in Endothelial Basal Medium-2 (EBM-2, Lonza) supplemented with 2% fetal bovine serum (FBS, Life Technologies). Plasma samples from each patient were mixed with TNF-α-blocking antibody (100ng/mL, Cell Signaling) or vehicle and incubated at 37C for 2 hours with gentle intermittent mixing to allow neutralization of the TNF-α in the plasma. After 2 hours, the cell culture media was aspirated from each well and the plasma samples with or without TNF-α-blocking antibody were added to each well. Cultures were then incubated for an additional 4 hours under standard conditions. After incubation, the cultures were aspirated and washed with PBS. RNA was isolated using a PureLink RNA Mini Kit (Life Technologies) and TF gene expression was measured by RT-PCR.

Assessment of Angiogenic Potential of Patients' Serum

Measurement of the angiogenic potential of each patient's serum was performed as we recently reported.[112] Briefly, serum samples from each patient were diluted 1:1 with EBM-2 with growth factor additives such that the final concentration of each exogenous growth factor in the serum/EBM-2 mixture was equal to that in EGM-2 (Lonza). TNF-α-blocking antibody (100ng/mL, Cell Signaling) or vehicle was mixed with each sample. The samples were then vortexed and incubated at 37C for 2 hours with gentle intermittent mixing to allow neutralization of the TNF-α in the serum. During this time, 24-well cell culture plates (Falcon) were coated

with Matrigel (Corning Life Sciences, Corning, NY) and allowed to solidify at 37 ºC. Cultured HUVECs were then washed with PBS, trypsinized, and centrifuged. Then, 200,000 HUVECs were resuspended in the serum/EBM-2 mixture from each patient with or without TNF-α-blocking antibody. This mixture was then gently pipetted into the Matrigel-coated wells and incubated overnight under standard conditions. Microtube formation was assessed by microscopy as described.[90] Total tube number in a low power field was quantified visually (5 fields per well were averaged) by blinded technicians.

Assessment of Rho Kinase Activity

Prior authors have shown other inflammatory factors besides TNF-α may be elevated after LVAD implantation.[64] We therefore hypothesized that Rho Kinase (ROCK) may also be elevated. We measured ROCK activity in these patients using the method we previously described.[118] Briefly, leukocytes were isolated from blood, protein was extracted, and ROCK activity was measured by western blot.

Measurement of Non-Surgical Bleeding Outcomes

Patients were monitored for NSB events for 1 year after sample collection, or until they received an OHT or died (if within 1 year). NSB was defined as gastrointestinal bleeding, intracranial hemorrhage, or epistaxis as previously described.[112]

Statistical Analyses

Statistical analyses were preformed using SPSS version 23.0. Continuous variables such as biomarkers were compared among the three cohorts using the Kruskal-Wallis test, followed by pairwise *post hoc* comparisons using the Mann-Whitney U test with Bonferroni adjustment when the omnibus test indicated a significant difference among the cohorts. For comparisons of continuous variables between two cohorts, the Mann-Whitney U test was used. Treatment conditions were compared within groups using the Wilcoxon Signed-Rank test. Categorical variables such as bleeding events were compared using Fisher's exact test. Clinical characteristics were compared using ANOVA or Student's t-test for continuous variables or Pearson Chi-Square testing for categorical variables as appropriate. Ordinal variables such as NYHA HF class were compared using the Wilcoxon Rank Sum test. Data are presented as mean ± standard deviation unless otherwise indicated. A 2-sided *P* value of <0.05 was considered statistically significant.

Results

TNF-α/Thrombin Synergy Regulates Endothelial Tissue Factor Expression

To investigate the effect of TNF-α and thrombin on TF expression in endothelial cells, we incubated HUVECs with TNF-α, Thrombin, TNF-α/Thrombin together, or control medium and measured TF gene expression by RT-PCR. TNF-α increased TF gene expression over 100-fold compared with control medium (103±20 RQ) while Thrombin did not (4±3 RQ). However, the combination of TNF-α/Thrombin further increased TF expression compared with either agent alone (177±19 RQ), Figure 3.1.

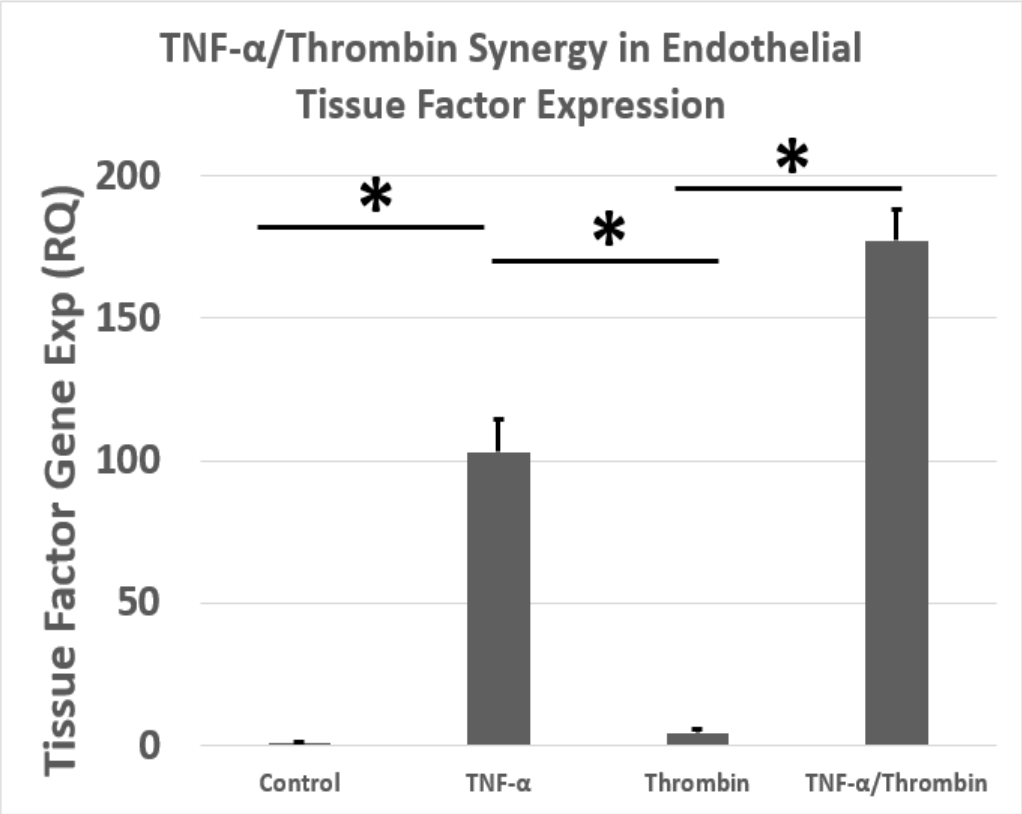


Figure 3.1: TNF-α/thrombin synergy drives endothelial TF expression. *Cultured HUVECs were incubated with control medium, TNF-α, thrombin, or TNF-α/thrombin together. TF gene expression was measured by RT-PCR. TNF-α induced TF gene expression more than control medium while thrombin did not. However, TNF-α/thrombin together induced more TF expression than either agent alone.*

TNF-α/Ang-2 Synergy Regulates Angiogenesis

To investigate the effect of TNF-α and Ang-2 on angiogenesis, we pre-incubated HUVECs with TNF-α or control medium, then incubated the cells on Matrigel with Ang-2 or control medium. As shown in Figure 3.2, TNF-α and Ang-2 induced more tubule formation (42.4±3.2 and 38.0±6.5 tubes per LPF respectively) than control medium (24.9±2.8 tubes per LPF), but TNF-α/Ang-2 together induced more tubule formation (51.6±4.8 tubes per LPF) than either agent alone.

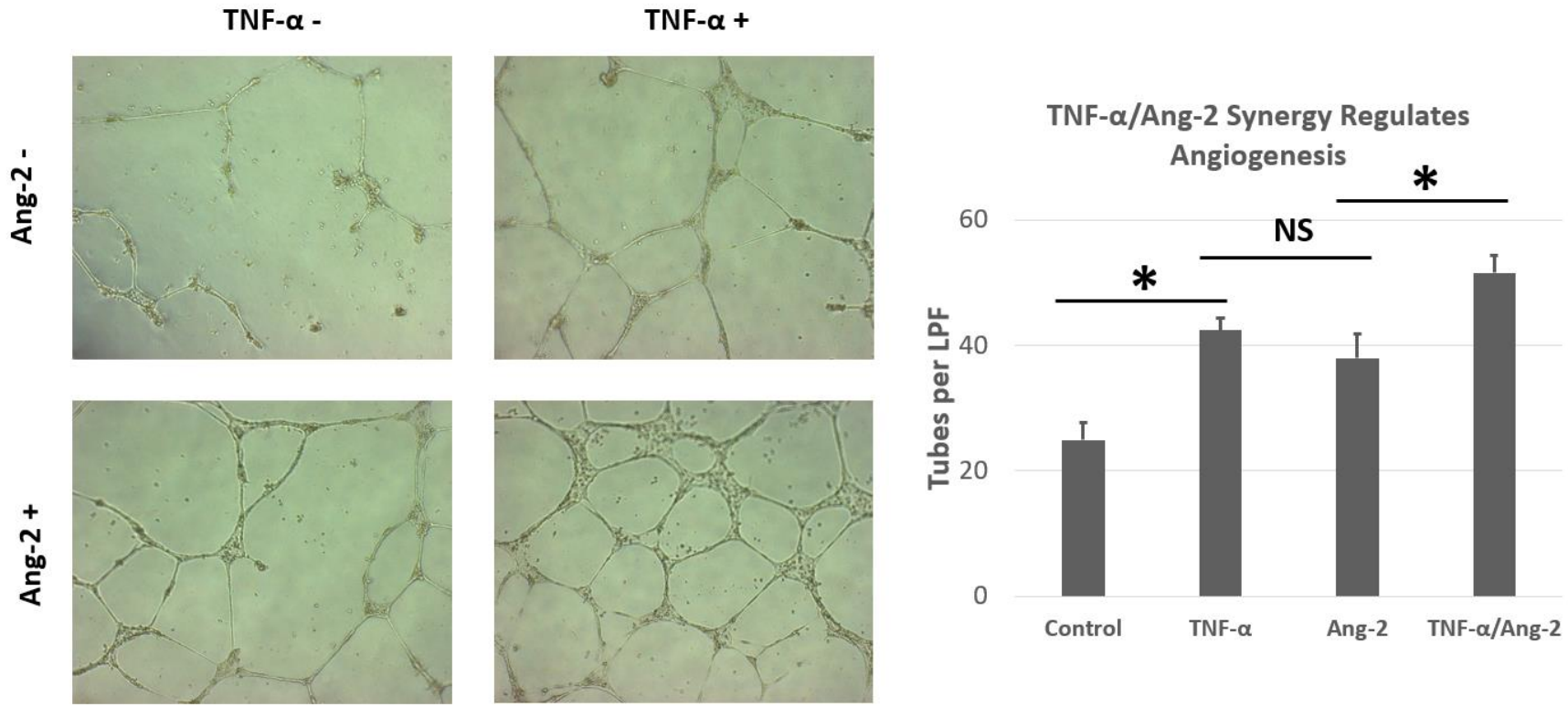


Figure 3.2: ***TNF-α/Ang-2 synergy drives angiogenesis.*** *Cultured HUVECs were pre-treated with/without TNF-α and then incubated on Matrigel with/without Ang-2. Tubule formation was measured by microscopy. TNF-α and Ang-2 both induced more tubule growth than control medium while the combination of TNF-α/Ang-2 induced more growth than either agent alone.*

TNF-α/Ang-2 synergy suppresses Ang-1 expression in pericytes

To investigate the effect of TNF-α and Ang-2 on Ang-1 expression in pericytes, we incubated cultured pericytes with TNF-α, Ang-2, TNF-α/Ang-2 together, or control medium and measured Ang-1 gene expression by RT-PCR. As shown in Figure 3.3, TNF-α suppressed Ang-1 gene expression compared with control medium (0.58±0.12 vs. 0.95±0.07 RQ respectively) while Ang-2 did not (1.15±0.10 RQ). TNF-α/Ang-2 together suppressed Ang-1 gene expression more than either agent alone (0.34±0.19 RQ).

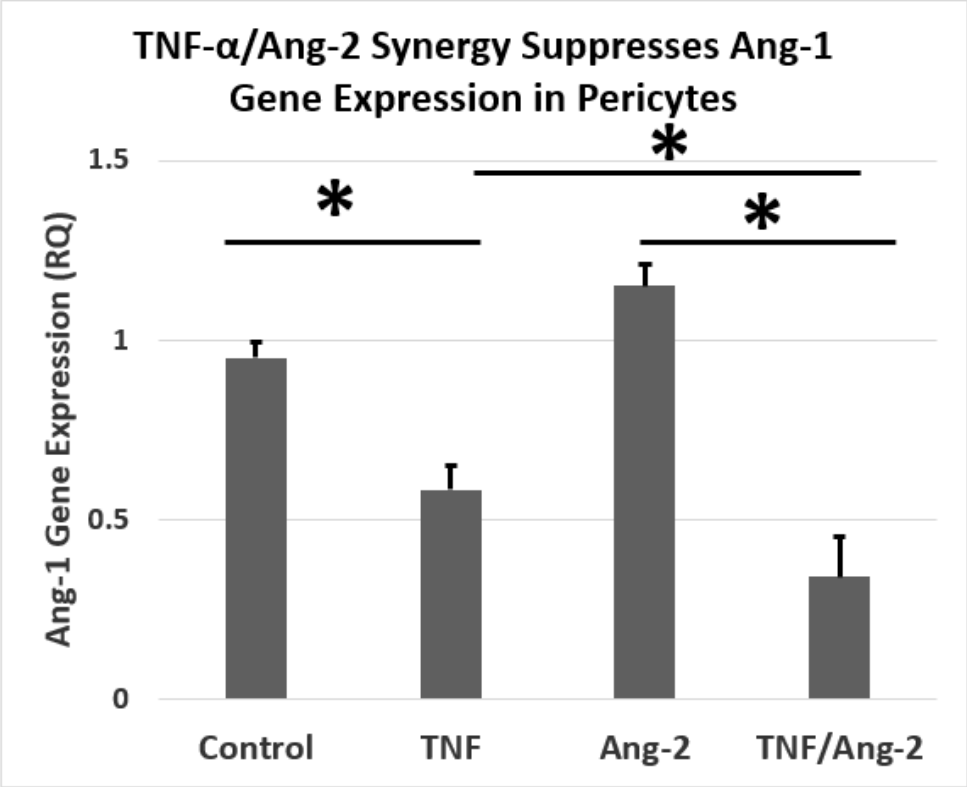


Figure 3.3: TNF-α/Ang-2 synergy suppresses Ang-1 expression in pericytes. *Cultured pericytes were treated with control medium, TNF-α, Ang-2, or TNF-α/Ang-2 together. Ang-1 expression was measured by RT-PCR. TNF-α suppressed Ang-1 gene expression compared with control medium while Ang-2 did not. TNF-α/Ang-2 together suppressed Ang-1 gene expression more than either agent alone.*

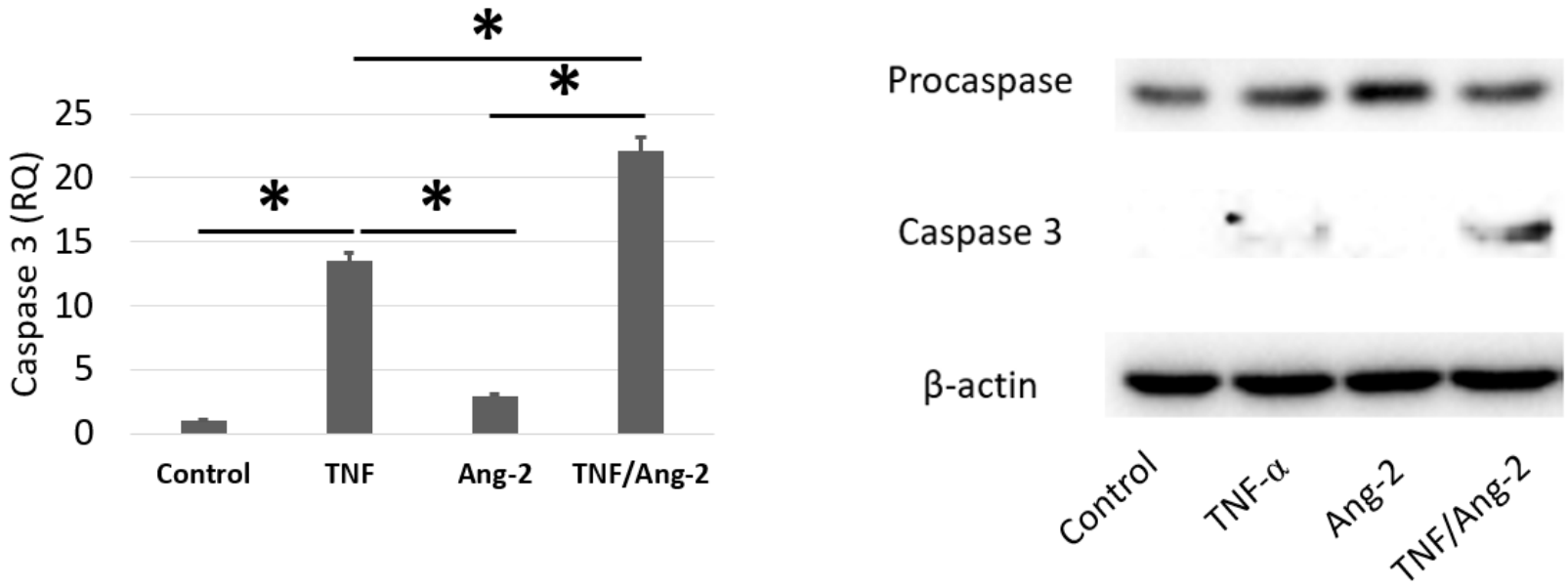


Figure 3.4: ***TNF-α/Ang-2 synergy regulates activation of Caspase-3 in pericytes.*** *Cultured pericytes were incubated with control medium, TNF-α, Ang-2, or TNF-α/Ang-2 together. Caspase-3 activation was measured by WB. TNF-α activated Caspase-3 more so than control medium while Ang-2 did not. TNF-α/Ang-2 together increased Caspase-3 activation more than either agent alone.*

TNF-α/Ang-2 synergy regulates pericyte apoptosis

To investigate the effect of TNF-α and Ang-2 on pericyte apoptosis, we incubated cultured pericytes with TNF-α, Ang-2, TNF-α/Ang-2 together, or control medium and measured the ratio of Caspase-3/Procaspase by western blot. As shown in Figure 3.4, TNF-α activated Caspase-3 more so than control medium (13.5±1.2 vs. 1.0±0.1 RQ respectively) while Ang-2 did not (2.9±0.4 RQ). TNF-α/Ang-2 together increased Caspase-3 activation more than either agent alone (22.1±2.9 RQ). We then incubated pericytes with TNF-α, Ang-2, TNF-α/Ang-2 together, or control medium and measured apoptosis by flow cytometry. As shown in Figure 3.5, TNF-α (0, 1, 5, 10, and 100 ng/mL) increased the percentage of apoptotic cells (Q3) in a dose-dependent manner (5.8±0.8, 12.1±2.6, 16.2±2.9, 21.4±9.1, and 22.0±4.7 %, respectively). As shown in Figure S6, TNF-α and Ang-2 increased the percentage of apoptotic cells (Q3) more so than control medium (10.3±2.2, 9.7±4.0, and 5.3±0.8 %, respectively). However, TNF-α/Ang-2 together increased apoptosis more than either agent alone (16.3±2.6). TNF-α also increased the percentage of dead cells (Q1+Q2) more so than control medium (1.69±0.10 vs. 0.79±0.09 %, respectively) while Ang-2 did not (0.68±0.31 %). The combination of TNF-α/Ang-2 (1.58±0.12) was not different from TNF-α alone.

Patient Enrollment

Adult patients in stable condition were recruited from the Advanced Heart Failure Clinic at the University of Chicago or from the cardiac catheterization laboratory if the patient had previously been seen in the Advanced Heart Failure Clinic. Three groups of patients were studied: Patients supported with an LVAD (n=32 Thoratec Heartmate II and 12 Heartware

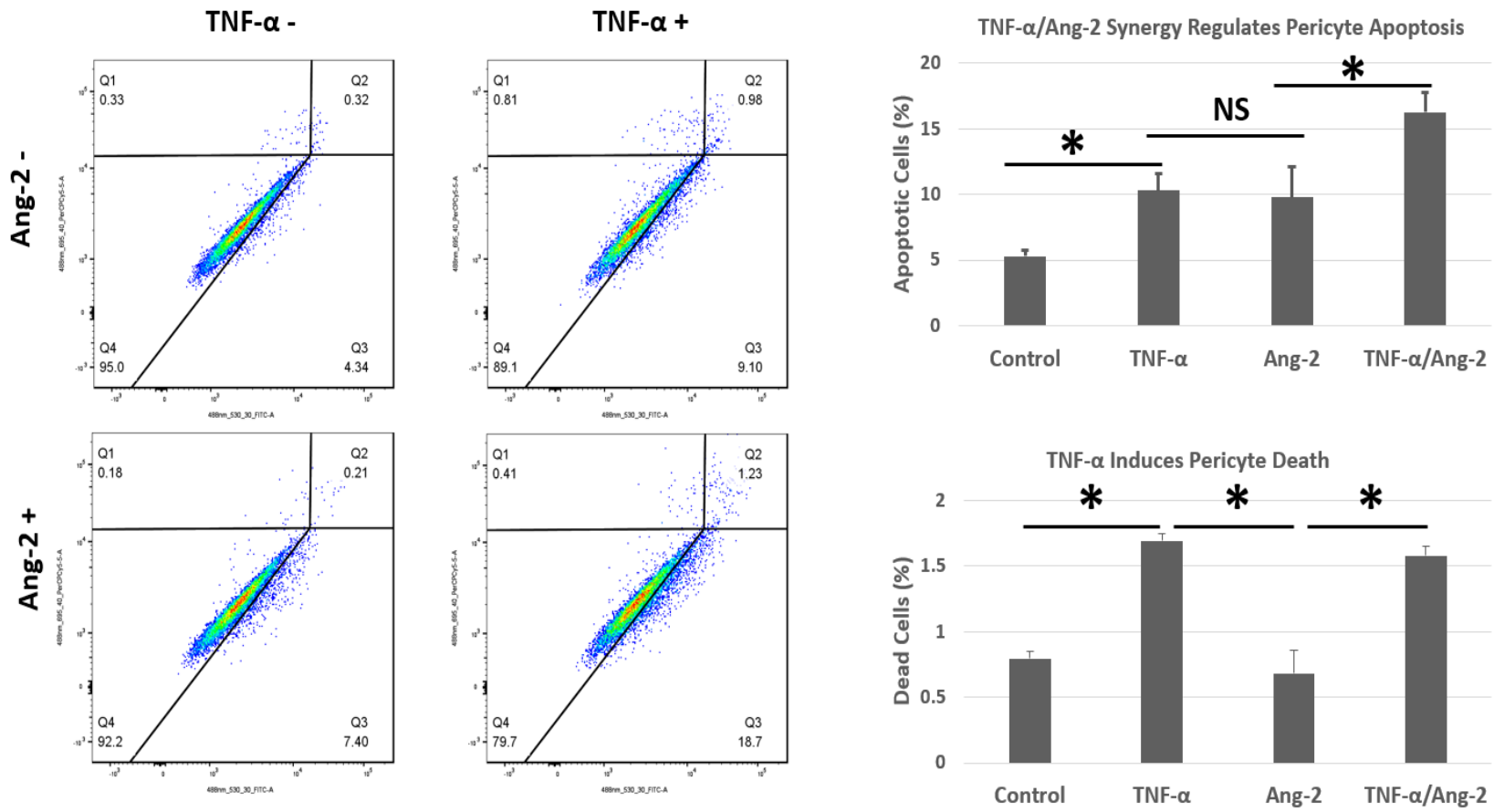


Figure 3.5: ***TNF-α/Ang-2 synergy regulates pericyte apoptosis.*** *Cultured pericytes were incubated with control medium, TNF-α, Ang-2, or TNF-α/Ang-2 together. Apoptosis was measured by flow cytometry as the percentage of cells in Q3 (lower right) and death was measured as the percentage of cells in Q1+Q2 (top). TNF-α and Ang-2 increased the percentage of apoptotic cells more so than control medium. However, TNF-α/Ang-2 together increased apoptosis more than either agent alone. TNF-α also increased the percentage of dead cells more so than control medium while Ang-2 did not. The combination of TNF-α/Ang-2 was not different from TNF-α alone.*

HVAD) at least 30 days post-implantation, functionally similar heart failure patients with reduced ejection fraction (HFrEF, LVEF <40%) without an LVAD, and patients with a history of OHT at least 30 days post-transplantation. OHT patients were included to control for the effects of increased cardiac output, general anesthesia, and sternotomy. All patients were in clinically stable condition at the time of enrollment and sampling. Patients were excluded if they had decompensated heart failure, active cancer within 1 year, untreated hypoxic conditions, acute thrombosis within 6 months, severe renal disease, or acute illness of any kind. All subjects were studied in the fasting state. As shown in Table 3.1, the groups were largely similar except in areas where differences would be expected (such as LVEF between HF and OHT patients). Despite efforts to enroll functionally similar HF and LVAD patients, the HF patients without an LVAD in this study were still slightly sicker (as measured by NYHA functional class), which was expected and theoretically could bias our study toward the null as worsening HF is known to increase systemic inflammation[88, 114] and associated biomarkers.[115-117] Subgroup comparisons

Table 3.1 – Clinical Characteristics				
	HF	**LVAD**	**OHT**	***p*-value**
Number of participants	32	44	25	
Age (years)	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
Days Post Implant (Median)	---	295.0±479.2	311.0±1257.9	0.1
eGFR (ml/min*1.73m^2)	68.7±20.6	59.6±24.5	65.8±21.9	0.208
NYHA HF Class (%)				<0.001
1	3	9	---	
2	22	70	---	
3	69	21	---	
4	0	0	---	
Pulse Pressure (mmHg)	49.6±15.7	29.8±8.8	47.0±9.7	<0.001
Statin (%)	44	59	96	<0.001
Warfarin (%)	44	93	4	<0.001
ACE-I/ARB (%)	84	46	12	<0.001
Anti-platelets (%)	53	90	52	<0.001

are shown in Tables 3.2-3.5.

Table 3.2 – Comparison of LVAD patients with High vs. Low TNF-α

	Low TNF-α	High TNF-α	P-value
N	23	21	
Age (years)	56.4±10.7	61.3±2.2	0.151
Days Post Implant	449±463	496±500	0.573
Pulse Pressure (mmHg)	29.7±10.0	29.8±7.6	0.773
eGFR (ml/min*1.73m^2)	61.0±24.7	58.1±24.8	0.742
LDH (U/L)	334.5±104.2	400.0±277.9	0.879
Hemoglobin (mg/dL)	12.0±1.6	10.9±1.6	0.032
Platelets (#/μL)	225.1±69.7	221.9±75.5	0.805
INR	1.9±0.6	1.8±0.4	0.637

Table 3.3 – Comparison of TNF-α in LVAD Patients Stratified by RV Dysfunction

	Normal	Mild	Moderate	Severe	Not Quantified
N	1	4	19	13	7
TNF-α (pg/mL)	0.57	5.91±2.09	6.05±5.61	6.16±3.59	6.36±2.40

Table 3.4 – Comparison of TNF-α in LVAD Patients Stratified by LVAD Type

	HM2	HVAD	p-value
N	32	12	
Speed (rpm)	9115±389	2746±138	n/a
Flow (Lpm)	5.4±1.2	4.6±1.3	n/a
Pulsatility Index	5.5±1.2	---	n/a
TNF-α (pg/mL)	5.35±3.98	7.46±5.23	0.142

Table 3.5 – Characteristics of LVAD Patients With and Without NSB within 1 Year

	Non-Bleeders	Bleeders	p-value
N	33	11	
Age (years)	58.3±10.5	60.1±11.7	0.689
% Female	30	18	0.698
Days Post Implant	414.3±420.3	643.7±605.1	0.196
Pulse Pressure (mmHg)	31.3±9.5	25.2±3.8	0.021
LDH (U/L)	347.0±147.0	100.0±330.1	0.810
Hemoglobin (mg/dL)	11.8±1.8	10.7±1.2	0.053
Platelets (#/μL)	224.7±71.7	220.4±75.1	0.728
INR	1.9±0.6	1.7±0.5	0.237
Antiplatelet use (%)	88	100	0.558
TNF-α (pg/mL)	5.3±4.9	7.9±1.9	0.005
Driveline Infection (%)	46	18	0.158

As shown, LVAD patients with high levels of TNF-α had slightly lower hemoglobin (Table 3.2) and TNF-α tended to rise with worsening right ventricular dysfunction (Table 3.3, as reported in clinically necessary echocardiography). There was no significant difference in TNF-α level between patients with Thoratec HeartMate2 and Heartware HVADs (Table 3.4). Between LVAD patients with and without NSB, there were no significant differences age, sex, duration of implantation, pulse pressure, lactate dehydrogenase, hemoglobin, international normalized ratio (INR), antiplatelet use, or occurrence of driveline infections (Table 3.5). However, TNF-α was significantly higher in LVAD patients with NSB.

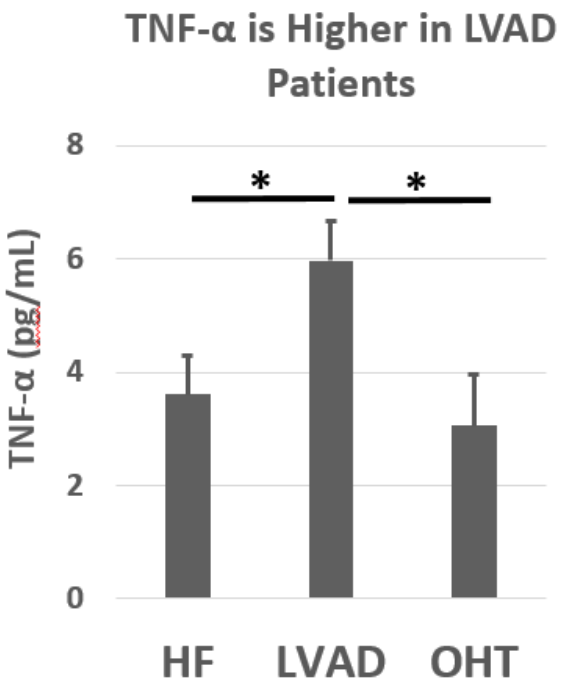


Figure 3.6: Plasma levels of TNF-α are elevated in LVAD patients. *TNF-α was measured in plasma from patients with HF, LVAD, or OHT. TNF-α was significantly higher in LVAD patients than HF or OHT.*

Elevated Circulating TNF-α in Plasma from LVAD Patients

To evaluate the circulating levels of TNF-α in patients with and without LVADs, we measured levels of TNF-α in platelet-poor plasma. As shown in Figure 3.6, TNF-α was significantly higher in patients with LVADs compared with HF or OHT (5.97±4.42, 3.61±3.31, 3.05±3.64 pg/mL respectively, omnibus $p<0.01$, HF vs LVAD $p<0.05$, LVAD vs OHT $p<0.05$,

HF vs OHT p=NS).

Elevated TNF-α in Serum from Patients with LVADs Suppresses Angiopoietin-1 Gene Expression in Pericytes

To investigate whether elevated TNF-α in serum from LVAD patients could suppress Ang-1 gene expression in pericytes, we incubated cultured pericytes with serum from patients with LVADs and stable HF without an LVAD. As shown in Figure 3.7, Ang-1 gene expression was significantly lower in pericyte cultures incubated with serum from LVAD patients compared with HF (0.63±0.09 vs. 0.75±0.12 RQ, p<0.05). This effect in the LVAD group was blunted by TNF-α blockade (0.80±0.09 RQ, p<0.01) while a non-significant increase was overserved in the HF group in response to TNF-α-blocking antibody (0.79±0.09, p=NS). Thus, while serum from LVAD patients suppressed Ang-1 expression more than serum from HF patients, Ang-1 expression was nearly identical in the two groups

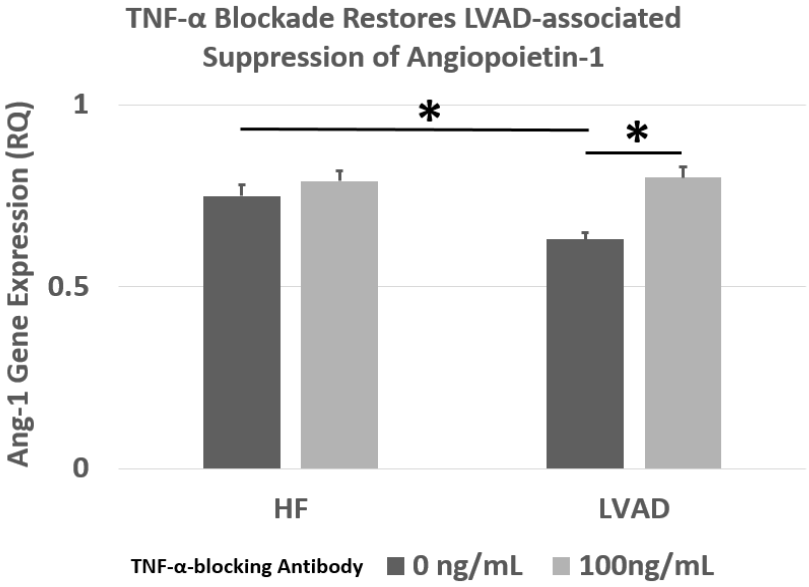


Figure 3.7: High TNF-α in serum from patients with LVADs suppresses Ang-1 expression in cultured pericytes. *Cultured pericytes were incubated with serum from patients with HF or LVAD in the presence/absence of TNF-α-blocking antibody. Serum from LVAD patients suppressed Ang-1 expression in the cultured pericytes compared with serum from HF patients and this effect was rescued with TNF-α-blockade.*

after TNF-α blockade.

Elevated TNF-α in Serum from Patients with LVADs Induces Pericyte Death

To investigate whether elevated TNF-α in serum from LVAD patients could induce pericyte death, we incubated cultured pericytes with serum from patients with and without LVADs. Pericyte death was significantly higher in cultures treated with serum from LVAD patients compared with HF or OHT (10.21±5.90, 5.27±7.03, 5.76±6.00 AU, omnibus $p<0.01$, HF vs LVAD $p<0.05$, LVAD vs OHT $p<0.01$, HF vs OHT p=NS, Figure 3.8) and this effect in the LVAD group was blunted by TNF-α blockade (6.91±4.99 AU, $p<0.001$). No significant difference was observed in the HF or OHT groups following TNF-α blocking antibody (5.99±5.44, 4.34±5.01 AU respectively, p=NS). Together, these findings suggest that high levels of TNF-α in patients with LVADs induce pericyte cell death and suppress expression of Ang-1.

Elevated Tissue Factor Protein Expression in Plasma and Freshly Isolated Endothelial Cells from Patients with LVADs

To evaluate the circulating levels of TF in patients with and without LVAD, we measured levels of TF in platelet-poor plasma. TF was significantly higher in the LVAD cohort compared with HF and trended higher than the OHT cohort as well (40.92±11.91, 31.74±12.59, 33.46±7.47 pg/mL respectively, omnibus $p<0.01$, HF vs LVAD $p<0.01$, LVAD vs OHT p=0.118, HF vs

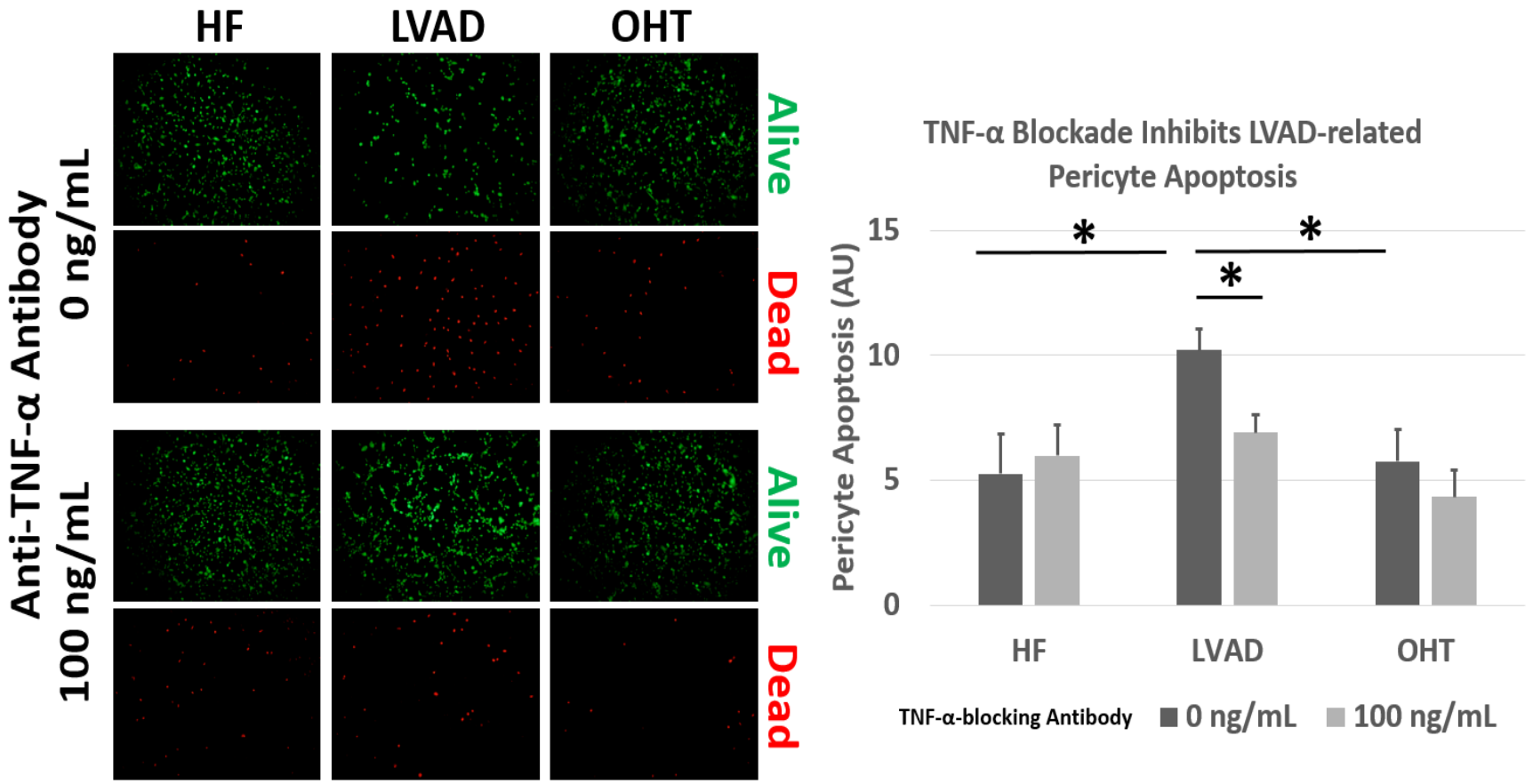
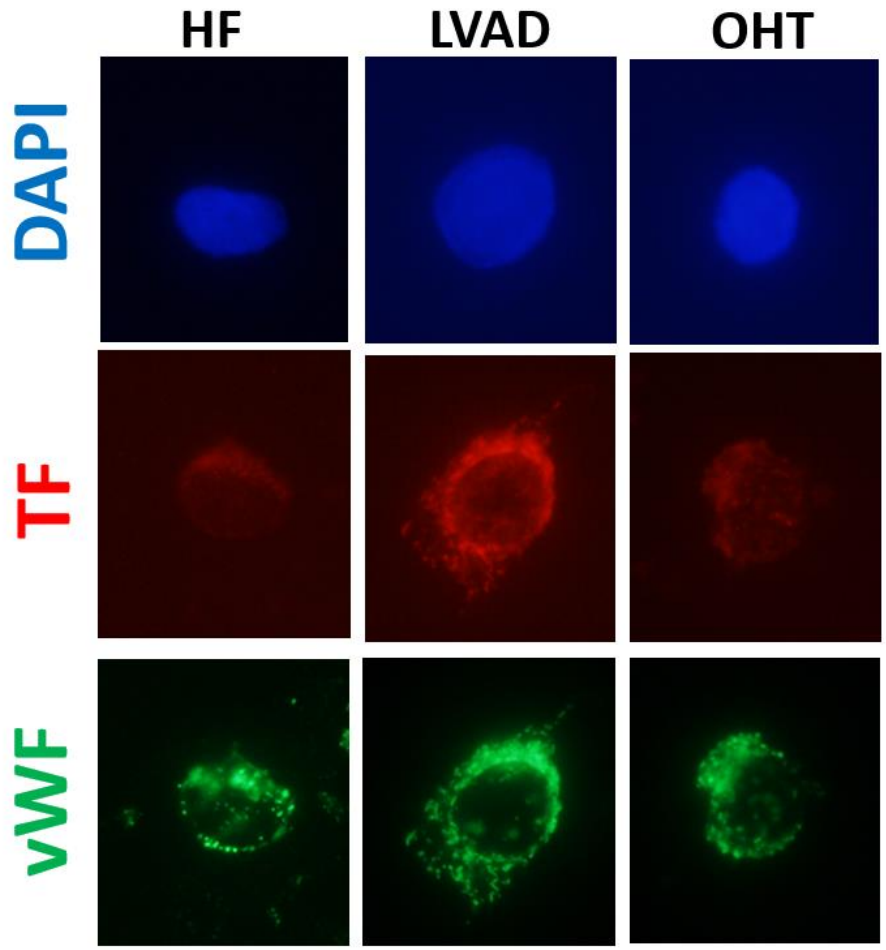


Figure 3.8: High TNF-α in serum from patients with LVADs induces pericyte cell death. *Cultured pericytes were incubated with serum from patients with HF, LVAD, or OHT in the presence or absence of a TNF-α-blocking antibody. Viability was assessed using a fluorescent viability kit. Cell death was measured as the ratio between live (green) and dead (red) cells. Pericyte death was significantly higher in cultures treated with serum from LVAD patients and this effect was blunted by TNF-α blockade.*



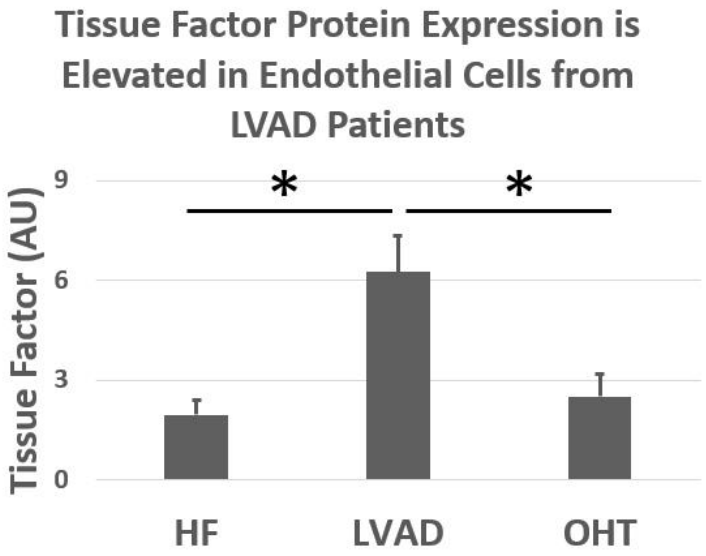


Figure 3.9: Endothelial Tissue Factor expression is elevated in LVAD patients. *Tissue Factor protein expression in freshly isolated endothelial cells from patients with HF, LVAD, or OHT was analyzed by quantitative immunofluorescence. Tissue Factor expression was significantly higher in endothelial cells from LVAD patients compared with HF or OHT.*

OHT, p=1.0). To investigate the source of the elevated circulating TF 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 TF in blood of patients with LVADs, TF protein expression in freshly isolated endothelial cells was also higher in patients with LVADs compared with HF or OHT (6.25±3.94, 1.95±1.37, 2.51±2.36 AU respectively, omnibus $p<0.01$, HF vs LVAD $p<0.05$, LVAD vs OHT $p<0.05$, HF vs OHT, p=1.0) (Figure 3.9). These findings suggest that over-expression of TF in the endothelium may be responsible for the elevated circulating TF levels in patients with LVADs.

Elevated TNF-α in Plasma from Patients with LVADs Increases Tissue Factor Gene Expression in Endothelium

To evaluate whether the increased TNF-α in plasma from patients with LVADs could be responsible for increased TF expression, we incubated HUVECs with plasma from each patient in the presence or absence of TNF-α blocking antibody and measured TF gene expression by RT-PCR. As shown in Figure 3.10, the plasma from patients with LVADs induced higher TF gene expression in the cultured endothelial cells compared with plasma from patients with HF or OHT (5.38±4.2, 1.52±0.96, and 2.03±1.49 RQ respectively, omnibus $p<0.01$, HF vs LVAD $p<0.01$, LVAD vs OHT $p<0.05$, HF vs OHT p=NS). In the LVAD cohort, this increased TF gene expression was significantly reduced with TNF-α blockade (2.88±1.22 RQ, $p<0.05$). In contrast, a non-significant decrease in TF gene expression in the presence of TNF-α blockade was noted in endothelial cells receiving plasma from patients with HF or OHT (1.18±0.96 and 1.33±0.79 RQ respectively, p=NS for both). Taken together, these data suggest that elevated

TNF-α in plasma from patients with LVADs induces increased endothelial TF expression.

Elevated TNF-α in Serum from Patients with LVADs Induces Angiogenesis

We have recently shown that high levels of Ang-2 in LVAD patients induce endothelial tube formation on Matrigel.[112] Prior studies have also shown that TNF-α increases endothelial tube formation on Matrigel in synergy with Ang-2.[119] To investigate whether the elevated TNF-α in

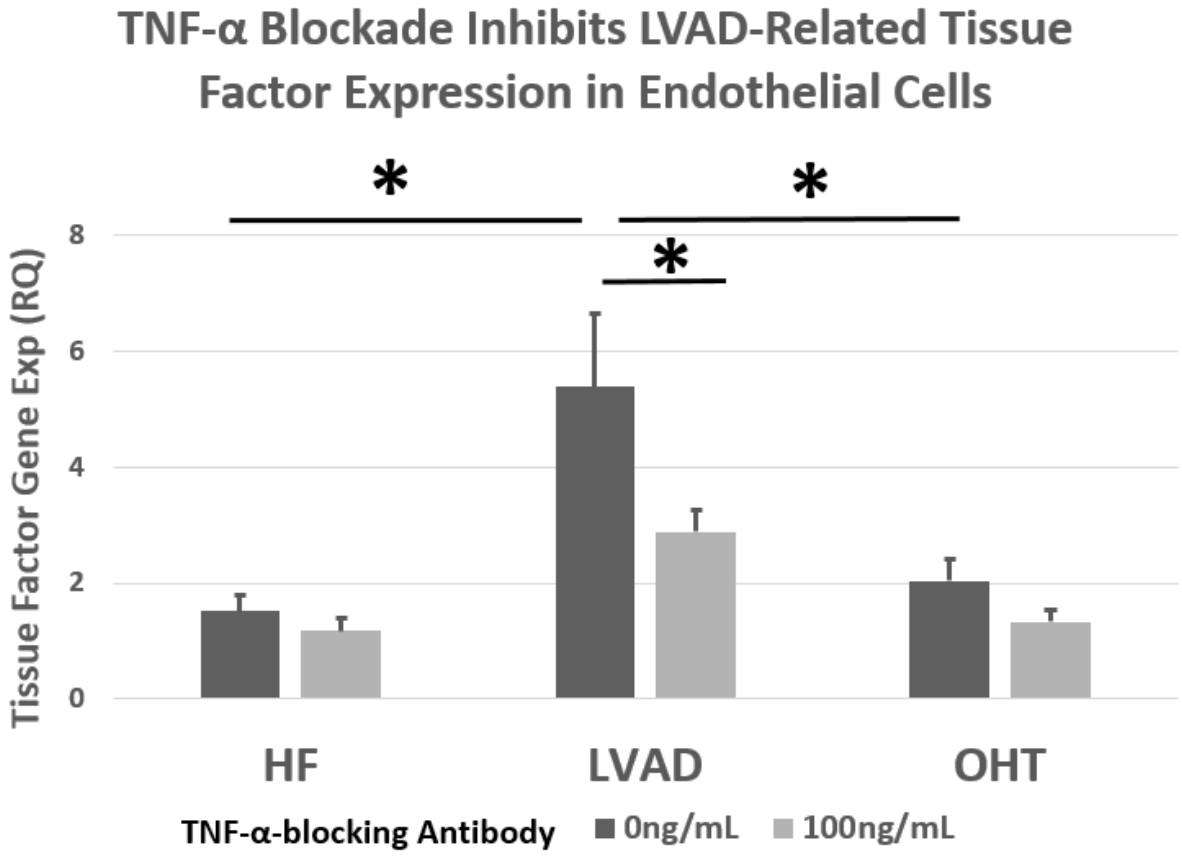


Figure 3.10: High TNF-α in plasma from patients with LVADs induces endothelial Tissue Factor expression. *Cultured HUVECs were incubated with plasma from patients with HF, LVAD, or OHT in the presence or absence of a TNF-α-blocking antibody. Tissue Factor gene expression was measured by RT-PCR. Plasma from LVAD patients induced more Tissue Factor gene expression than plasma from HF or OHT patients and this effect was blunted by TNF-α blockade.*

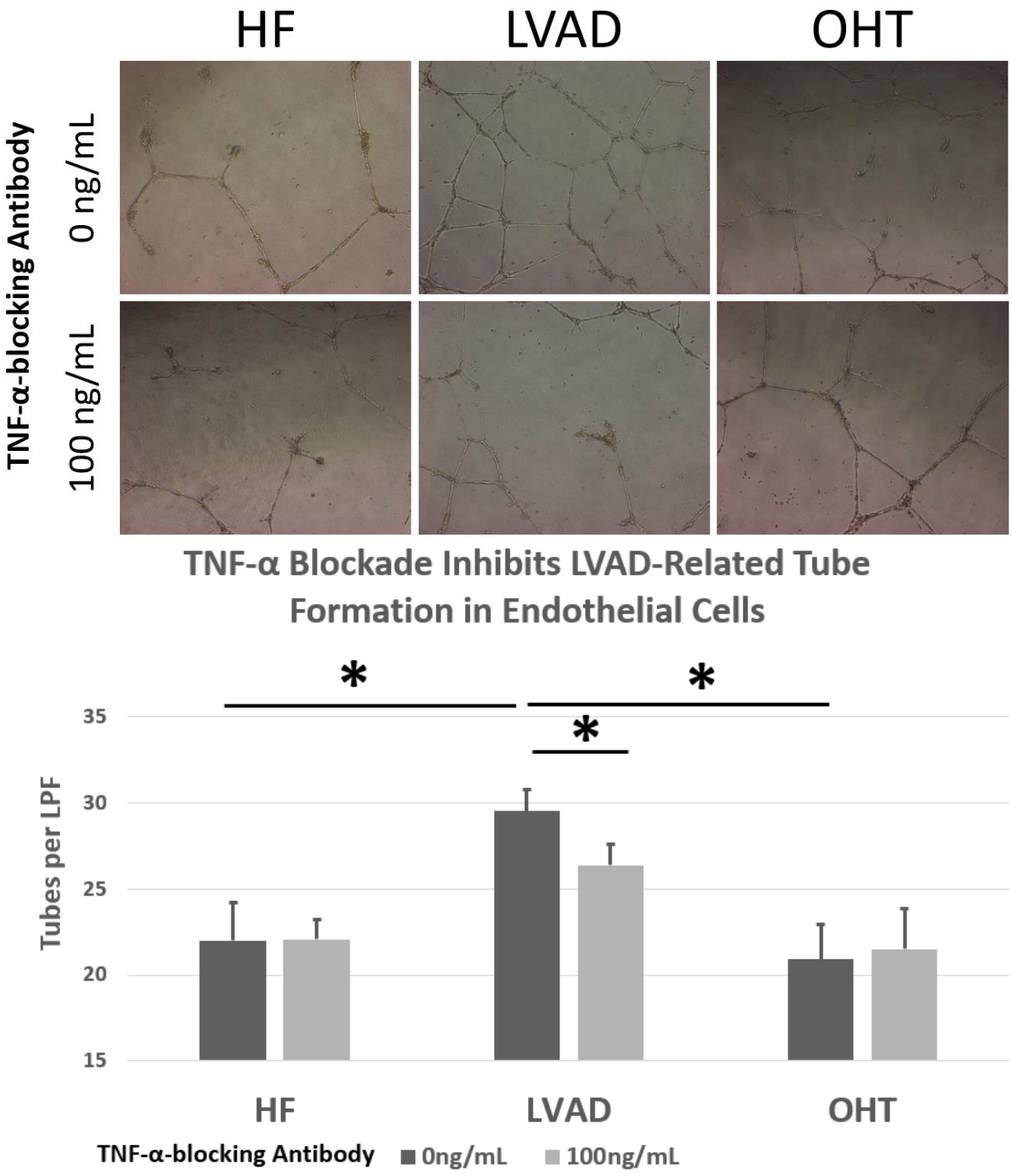


Figure 3.11: High TNF-α in serum from patients with LVADs induces angiogenesis in human endothelium. *HUVECs were assayed on Matrigel with serum from patients with HF, LVAD, or OHT in the presence or absence of a TNF-α-blocking antibody. Tube formation was significantly higher in cultures treated with serum from LVAD patients and this effect was blunted by TNF-α blockade.*

serum from patients with LVADs contributes significantly to endothelial tube formation, we incubated HUVECs grown on Matrigel with serum from patients with LVAD, HF, or OHT in the presence or absence of a TNF-α-blocking antibody. Serum from patients with LVADs induced more tubule formation than did serum from patients with HF or OHT (29.52±7.06, 22.00±6.94, and 20.92±8.14 tubes per low power field respectively, omnibus $p<0.01$, HF vs LVAD $p<0.05$, LVAD vs OHT $p<0.01$, HF vs OHT p=NS) (Figure 3.11). This effect was blunted in LVAD patients by the TNF-α blocking antibody (26.42±6.83 tubes per low power field respectively, $p<0.05$), indicating that elevated TNF-α in the serum from patients with LVADs contributes to increased tubule formation. No significant difference was observed in the HF or OHT groups in response to the TNF-α blocking antibody (22.07±3.69 and 21.50±9.37 tubes per low power field respectively, p=NS for both).

Elevated ROCK Activity in LVAD Patients

We measured ROCK activity in patients with/without LVADs. As shown in Figure 3.12, ROCK activity was significantly higher in LVAD patients compared with HF or OHT (2.26±1.77, 1.19±1.01, and 1.02±0.77 RQ respectively, omnibus $p<0.01$, HF vs LVAD $p<0.05$, LVAD vs OHT $p<0.05$, HF vs OHT p=NS).

The Combination of Elevated TNF-α and Ang-2 in Patients with LVADs is Strongly Associated with an Increased Risk of Non-Surgical Bleeding Events

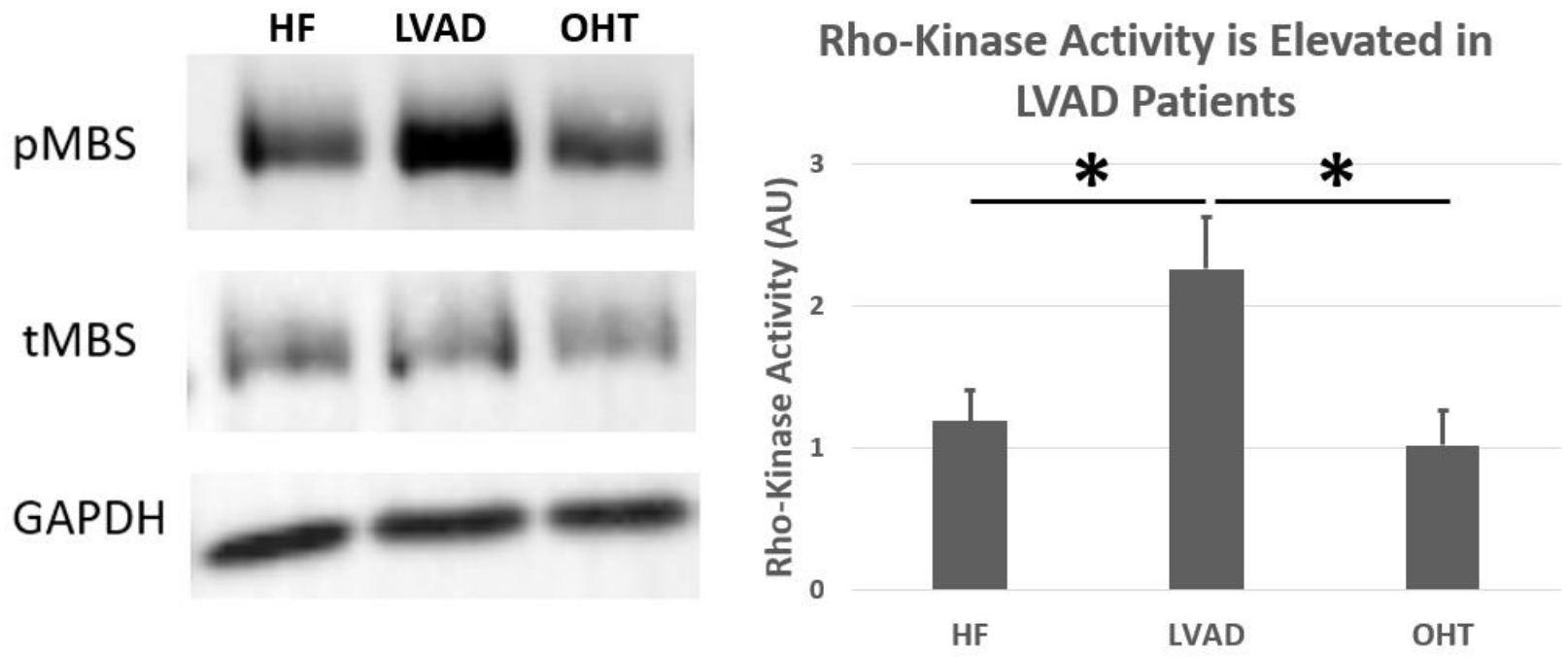


Figure 3.12: ROCK activity is elevated in LVAD patients. *Leukocyte ROCK activity was measured by western blot as the ratio between phospho-Myosin Binding Subunit (pMBS) and total Myosin Binding Subunit (tMBS). ROCK activity was higher in LVAD patients than in HF or OHT patients.*

We have recently shown that elevated Ang-2 is associated with bleeding events in LVAD patients.[112] However, elevated Ang-2 alone was not fully predictive of bleeding events. To investigate whether TNF-α predicts NSB events in LVAD patients, we reviewed the medical records of LVAD patients in this study. Within 1 year of sample collection, 11 patients experienced NSB, defined as previously reported.[112] Patients who bled within 1 year had significantly higher TNF-α levels than non-bleeders (7.9±1.9 vs. 5.3±4.9 pg/mL respectively, $p<0.01$). Among LVAD patients with TNF-α levels above the mean, the 48% experienced NSB within 1-year of sample collection (n=10/21) compared with 4% (n=1/23) in patients with TNF-α below the mean ($p<0.01$). Among LVAD patients with both TNF-α and Ang-2 above the mean,

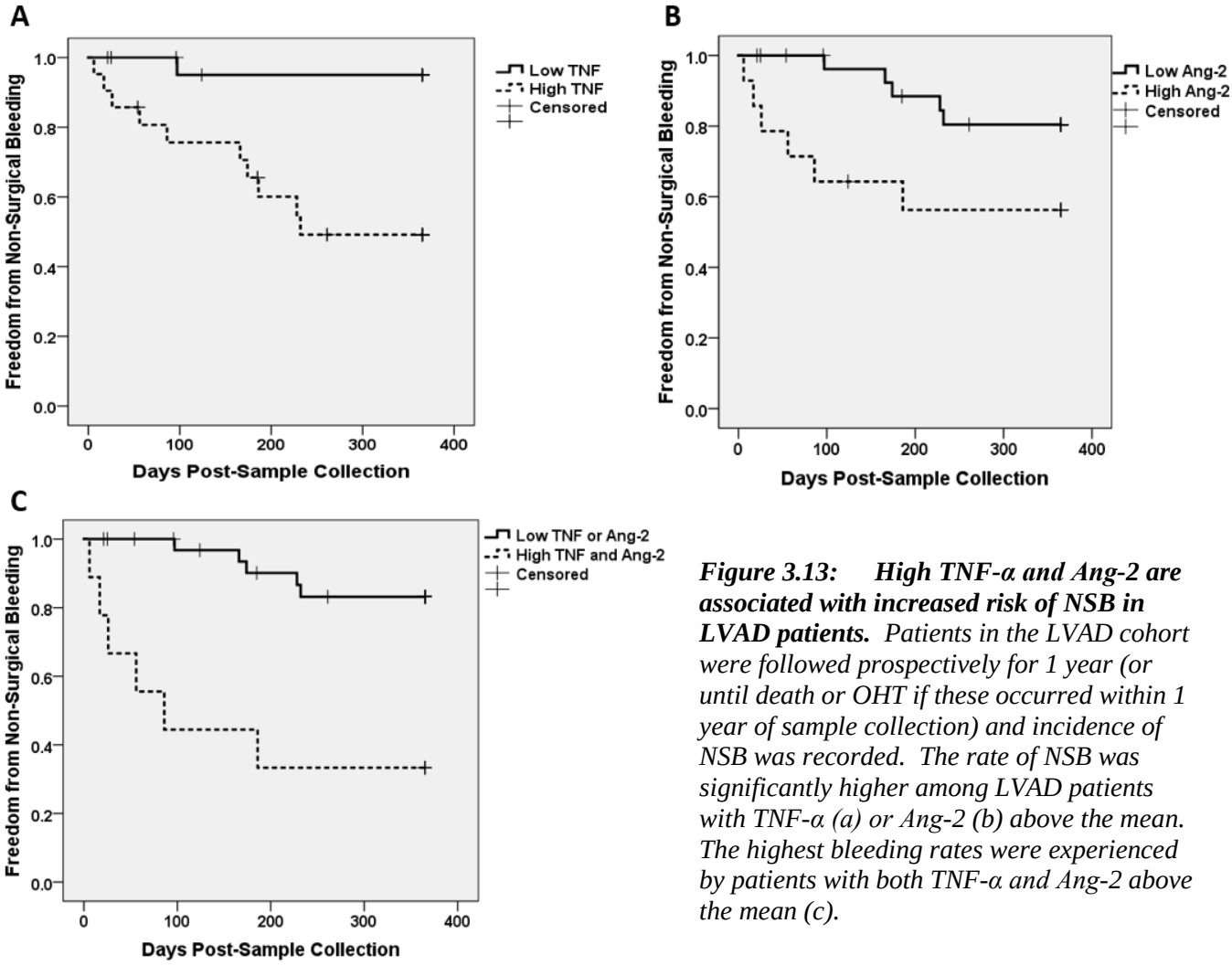


Figure 3.13: High TNF-α and Ang-2 are associated with increased risk of NSB in LVAD patients. *Patients in the LVAD cohort were followed prospectively for 1 year (or until death or OHT if these occurred within 1 year of sample collection) and incidence of NSB was recorded. The rate of NSB was significantly higher among LVAD patients with TNF-α (a) or Ang-2 (b) above the mean. The highest bleeding rates were experienced by patients with both TNF-α and Ang-2 above the mean (c).*

67% experienced NSB within 1-year (n=6/9) compared with 14% (n=5/35) in patients with one or both biomarkers below the mean ($p<0.01$). Kaplan-Meier Distributions are shown in Figure 3.13a-c.

Discussion

In this study, we evaluate the role of TNF-α in LVAD patients in the activation of angiogenic and thrombotic pathways compared with HF or OHT patients. Our main findings are the following: (1) Patients with LVADs have higher circulating levels of TNF-α; (2) These higher levels of TNF-α induce apoptosis of pericytes and suppress Ang-1 expression, which may contribute to a decline in the Ang-1/Ang-2 ratio observed in our LVAD patients; and (3) Circulating levels of TF and endothelial TF expression are higher in LVAD patients and high levels of TNF-α in LVAD patients induce TF expression in endothelial cells. As TF is a potent activator of thrombin, which is elevated in LVAD patients,[112] our data suggest that TNF-α may indirectly contribute to thrombin activation in LVAD patients. While our prior findings report the role of high levels of thrombin in LVAD patients driving endothelial Ang-2 expression and angiogenesis,[112] the findings in the current study further identify a likely mechanism behind the elevation of thrombin and loss of Ang-1 in these patients. Further, we found that high levels of TNF-α in LVAD patients increase angiogenesis *in vitro* in a fashion similar to Ang-2.[112] These results suggest that the synergic effect of TNF-α and Ang-2 in promoting endothelial inflammation[113] and abnormal angiogenesis as previously described in tissue culture[119] and mouse models[55] is likely driving angiodysplasia and vascular instability in LVAD patients. These findings are accompanied by elevation of ROCK activity, a mediator of endothelial

inflammation[120] also associated with vascular instability.[121] Finally, we found that high levels of TNF-α confer an increased risk of NSB while elevation of both TNF-α and Ang-2 together further compounds this risk.

Our findings help explain the decrease in plasma levels of Ang-1, which we previously observed in LVAD patients.[112] Ang-1 is necessary to maintain the integrity and stability of blood vessels, and a low Ang-1/Ang-2 ratio can lead to vascular inflammation and abnormal vessel growth. Indeed, increased expression of Ang-2 with a corresponding decrease in Ang-1 is associated with vascular malformations,[31] gastrointestinal angiodysplasia,[32] and increased capillary density.[33] Histologically, angiodysplasia is associated with loss of pericyte coverage of proliferating endothelial cells.[34, 35] Prior studies have suggested that TNF-α/Ang-2 synergy is responsible for pericyte dropout and apoptosis in diabetes.[58, 59] We have shown that TNF-α-induced pericyte apoptosis in LVAD patients is also associated with a substantial suppression of Ang-1 expression and that this effect was blunted by TNF-α blockade.

Studies in tissue culture[119] and animal models[55] have implicated the synergic effect of TNF-α and Ang-2 on angiogenesis. TNF-α primes the sprouting endothelial tip cells to receive the angiogenic signal from Ang-2 and other angiogenic growth factors such as VEGF and PDGF.[122] In LVAD patients, our findings suggest that elevation of TNF-α confers a substantial increase in NSB risk which is augmented in concert with high levels of Ang-2. TNF-α also regulates blood vessel remodeling and lymphangiogenesis in rodent models.[123] Interestingly, other disease states where TNF-α is elevated such as rheumatoid arthritis have also been associated with pathologic angiogenesis.[124] Notably, the effect of TNF-α on angiogenesis appears to be paradoxical and dose-dependent such that increasing doses of TNF-α induce increasingly more vessel growth until a boundary is reached where the effect is abolished and

toxicity follows.[119, 125] Together these findings may help explain the complex relationships between TNF-α, Ang-2, and NSB.

Finally, our findings further link LVAD-related angiodysplasia with thrombosis. We have recently shown that high levels of thrombin in LVAD patients lead to Ang-2 over-expression.[112] Here, we demonstrate that TF is significantly higher in LVAD patients, both in the blood and endothelium and that high levels of TNF-α in LVAD patients are likely responsible for TF over-expression. Further, the synergic effect of TNF-α and thrombin on TF

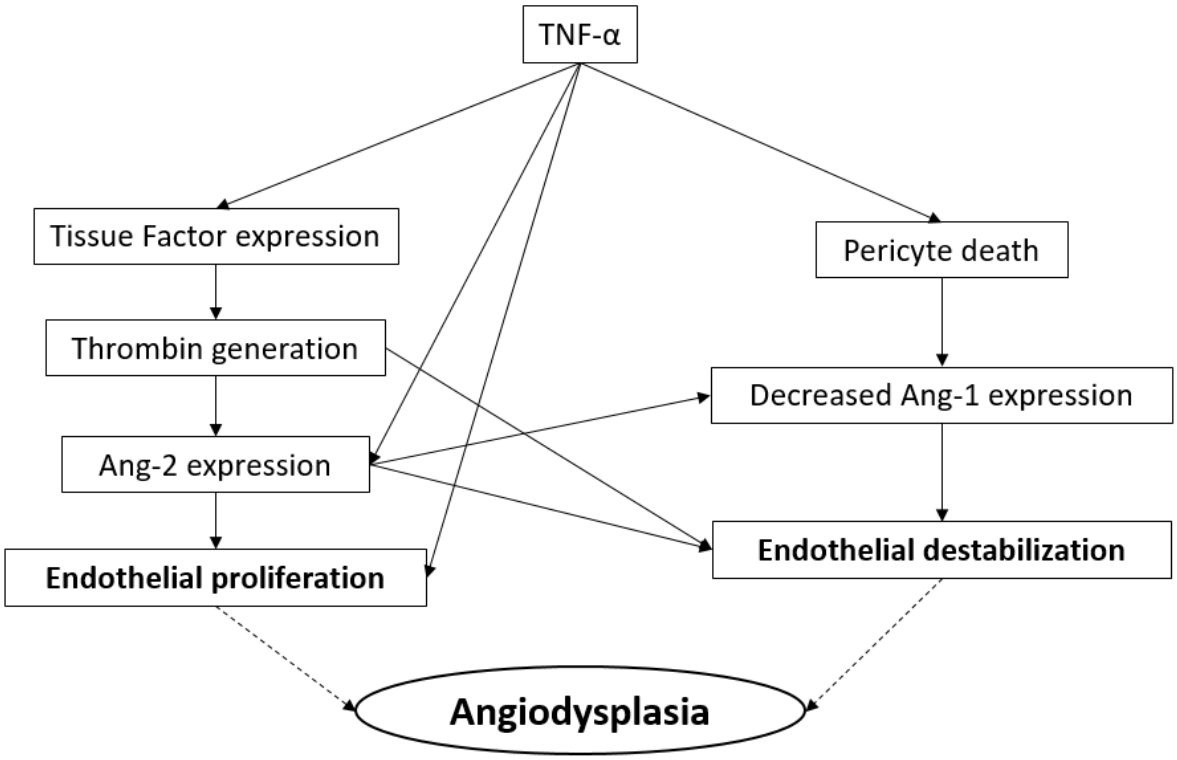


Figure 3.14: Proposed model of TNF-α as a central regulator of LVAD-related angiodysplasia. *TNF-α induces endothelial TF expression which generates thrombin which in turn induces endothelial Ang-2 expression. TNF-α also directly stimulates Ang-2 expression. Together, TNF-α and Ang-2 promote endothelial proliferation. TNF-α also induces pericyte apoptosis/death which leads to decreased Ang-1 expression and endothelial destabilization which is augmented by Ang-2 and thrombin. Together, endothelial proliferation and destabilization and loss of pericyte coverage lead to angiodysplasia.*

expression in the endothelium[61-63] likely leads to a feed-forward response, which produces more thrombin. This may lead to over-expression of Ang-2 as we have shown[112] and/or thrombosis.

The results of this study extend our current understanding of the pathogenesis of LVAD–related angiodysplasia by demonstrating the central role of TNF-α in regulating vessel stability and growth in LVAD patients (Figure 3.14). Our findings complement our prior work implicating Ang-2 as a potent promoter of vessel growth in LVAD patients as these two factors likely work in synergy in LVAD patients to enhance the development of angiodysplasia. Further, these findings also suggest that therapeutic inhibition of TNF-α and Ang-2 together may be useful in preventing angiodysplasia in LVAD patients. Indeed, such a strategy was successful in preventing pathologic angiogenesis in the lungs of infected mice.[55] As many TNF-α inhibitors are commercially available and many Ang-2 inhibitors are currently in development, further study will be needed to determine if such therapeutic strategies are safe and effective for LVAD patients.

Our study has several limitations. While we have shown that inhibition of TNF-α *in vitro* inhibits many of the pathologic effects of TNF-α on vascular cells *ex vivo*, the effect of inhibiting TNF-α in LVAD patients is not known and remains to be studied. The freshly isolated endothelial cells isolated from the LVAD patients in this study are vena caval in origin, while it is likely the capillary endothelial cells that contribute to angiodysplasia in the GI tract. However, TNF-α, Ang-2, and thrombin are all circulating freely and are able to interact with all vascular beds. It is likely that these systemic factors induce effects on vascular beds remote from their origin. Still, we cannot discount a localized effect from TF, Ang-2, and/or TNF-α over-expression in the small vessels of the gut. Similarly, the reason behind the predilection of LVAD-related angiodysplasia for the GI tract and nasopharynx remains unknown. It is also not

possible to account for all confounding variables in human studies. However, we sought to minimize confounding through the enrollment of well-matched control groups including OHT patients to control for the effects of increased flow, sternotomy, and general anesthesia. We acknowledge that OHT patients are treated with immunosuppressants which are not used on LVAD patients. While the effects of these drugs on the markers in this study are not known, we have attempted to address this issue by enrolling two control groups, patients with OHT and patients with stable HF who are not taking immunosuppressants. Finally, this study was designed to describe the central role of TNF-α in regulating vascular pathologies in LVAD patients. We acknowledge that blood flow conditions differ markedly between patients with and without and LVAD. While evidence suggesting a link between flow conditions and bleeding exists,[16] the effect of this altered flow on inflammation and biomarker expression is not known. While the combination of high TNF-α and Ang-2 was strongly associated with NSB in this study, the relationship between these markers and angiodysplasia was not directly addressed. Nevertheless, all patients who experienced NSB in our study were later found to have angiodysplasia on endoscopy. We are however unable to determine the prevalence of subclinical angiodysplasia in this group, as patients who did not experience bleeding did not undergo endoscopy.

Conclusions

TNF-α is a central regulator of vascular instability and NSB in LVAD patients and likely acts in synergy with Ang-2 and thrombin to augment its effects. Further study is needed to determine whether TNF-α blockade could prevent complications in LVAD patients.

Chapter 4

The Butyrate-Producing Gastrointestinal Microbiome and Non-Surgical Bleeding in Human Patients with Continuous-Flow Left Ventricular Assist Devices: A Pilot Study

Abstract

Background: Angiogenic and inflammatory biomarkers are linked with gastrointestinal bleeding in patients with LVADs. However, it is not known why bleeding occurs in only 20-30% of LVAD patients and why the overwhelming majority of bleeding events arise from the GI tract. Butyrate, produced by specific gastrointestinal microbes, potently inhibits the expression and action of several angiogenic and inflammatory molecules and inhibits angiogenesis *in vitro* and *in vivo*. We hypothesized that LVAD patients who bleed may lack specific butyrate-producing gut microbes.

Methods: Forth-five consecutive LVAD patients admitted to the medical ward at UCMC were enrolled and asked to provide 3 stool samples which were collected by nursing staff. Genomic DNA was extracted from each sample. Relative populations of butyrate-producing microbes were determined by RT-PCR. The medical record was queried to determine the patient's bleeding history. Fecal occult blood testing (FOBT) was performed on each sample. To determine whether plasma from LVAD patients with GI bleeding induces endothelial destabilization, HUVECs were incubated with plasma from each patient and endothelial contraction force was measured by traction force microscopy.

Results: Patients with active GI bleeding at the time of sample collection had lower relative butyrate-producing microbes than patients who had bled during prior hospitalizations but were not currently bleeding, and these were lower than patients who never had bled. Similarly, patients who had any history of bleeding had lower butyrate-producing microbes than patients who had never bled, and patients who were bleeding currently had lower butyrate-producing microbes than those who were not currently bleeding. Patients with a positive fecal occult blood test (FOBT) had lower butyrate-producing microbes than those with a negative FOBT. Plasma from LVAD patients with NSB induced more endothelial cell contraction force than did plasma from non-bleeders.

Conclusions: LVAD patients with gastrointestinal bleeding may be deficient in butyrate-producing microbes. This deficiency is associated with increased endothelial cell contraction force.

Introduction

We have recently shown that Angiopoietin-2 (Ang-2) and Tumor Necrosis Factor-α (TNF-α) are critical drivers of LVAD-related angiodysplasia and high circulating levels of these markers predict non-surgical bleeding events in LVAD patients.[112, 126] TNF-α in LVAD patients induce pericyte apoptosis which results in low Ang-1 levels and a decline in the Ang-1/Ang-2 ratio. Further, TNF-α promotes abnormal angiogenesis in synergy with Ang-2[55] and regulates Ang-2 expression in cell culture models.[56] TNF-α also induces vascular leakage[60] and promotes surface expression of Tissue Factor (TF, Factor III) in endothelial cells.[61] TF produces thrombin in the plasma which in turn increases expression and release of Ang-2 from the endothelium.

These effects are augmented in synergy with thrombin itself.[62, 63] In this way, Ang-2 and TNF-α are intimately linked and act together to drive angiodysplasia. Consistent with this, we found that LVAD patients with high blood levels of both Ang-2 and TNF-α have a dramatically elevated risk of NSB, approaching 70% after 1 year, while patients with normal levels of either marker bleed significantly less.[126] Therefore, inhibition of Ang-2/TNF-α could treat or prevent LVAD-related NSB.

However, the reason why some LVAD patients have elevated Ang-2 and TNF-α and ultimately bleed and others do not is unknown. Further, the predilection for the gastrointestinal tract as the primary site of bleeding among LVAD patients remains unexplained. Prior studies suggest significant differences in bleeding risk among patients with certain demographics such as older age, female sex, and other factors.[5] Since many of these factors are associated with changes in the gastrointestinal microbiome, we hypothesized that differences in the microbiome could pre-dispose patients to NSB.

Butyrate is a 4-carbon short chain fatty acid (SCFA) that strongly inhibits the expression of both TNF-α[70] and Ang-2[71] and blunts TNF-α/Ang-2-dependent vascular destabilization.[29, 72-75] Butyrate is produced through fermentation of dietary fiber by specific gut microbes including *Eubacterium rectale/Roseburia spp*. and *Faecalibacterium prausnitzii*.[68, 69] Oral butyrate supplements are widely available and butyrate treatment reduces gut inflammation and angiogenesis in humans and animal models.[76-81] However, dietary fiber restrictions and antibiotic treatment, conditions common in LVAD patients, cause loss of butyrate-producing microbes (dysbiosis) and lower circulating butyrate levels which lead to vasculopathy.[82] Taken together, loss of butyrate-producing microbes in LVAD patients may lead to the over-expression of TNF-α and Ang-2 and the development of angiodysplasia in the gut. However, the interplay

among the butyrate-producing microbiome, TNF-α, and Ang-2 has not been explored in relation to LVAD-related NSB. We hypothesized that abundance of butyrate and butyrate-producing microbes are lower in the stool of LVAD patients with NSB vs. non-bleeders.

We have also recently shown that Rho-kinase (ROCK) activity is elevated in LVAD patients,[126] particularly among those with NSB. ROCK is a well-known mediator of endothelial inflammation[120] and mediates vascular instability and endothelial retraction.[121] TNF-α and Ang-2 exert many of their effects on endothelial retraction and permeability through the Rho/ROCK pathway.[65-67] Since TNF-α and Ang-2 are elevated in LVAD patients with NSB, we hypothesized that TNF-α/Ang-2 synergy induce vascular destabilization in LVAD patients with NSB and that inhibition of this pathway could prevent vascular destabilization in LVAD patients.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and grown using Endothelial Growth Medium-2 (EGM-2, Lonza) on T-75 flasks (Falcon). All cultures were grown under standard conditions (37 ºC, 5% CO_2). Cells were grown to 70% confluence, washed in PBS, trypsinized, and passaged. Cultured cells were used for all experiments prior to passage 7.

Measurement of the Effect of Butyrate on TNF-α/Ang-2-induced Angiogenesis

To investigate the effect of butyrate on TNF-α/Ang-2-induced angiogenesis, we incubated HUVECs with TNF-α (10ng/mL) in DMEM with 10% FBS or control medium (DMEM with 10% FBS) for 48 hours. Cells were then rested overnight in DMEM with 10% FBS. Cells were then trypsinized and resuspended in EBM-2 with 20% FBS with or without Ang-2 (100ng/mL) and with or without Butyrate 2.5 mM. This mixture was then pipetted into Matrigel (Corning) coated 24-well plates and incubated overnight under standard conditions. Tubule formation was then measured by microscopy.

Study Subjects

A cross sectional study was performed. We enrolled LVAD patients (Thoratec Heartmate II or Heartware HVAD) at least 30 days post-implantation admitted to UCMC with NSB. LVAD patients with no history of NSB admitted with other problems (arrhythmias, ICD shocks, etc) served as controls. Patients were excluded from the study if they had were currently receiving intravenous antibiotics, if they had known infectious processes in the GI tract such as clostridium difficile colitis, or severe renal disease defined as an estimated glomerular filtration rate (eGFR) less than 30 ml/min*1.73m^2. Clinical information was obtained from the medical record. LVAD parameters were obtained from the LVAD control module. All blood was collected in the fasting state. Stools were collected as they became available. We collected the first 3 stools from each patient. The stool samples were immediately frozen at -20C using special freezers on the hospital ward, collected daily by study staff, transported back to the laboratory. The study protocol was approved by the University of Chicago Institutional Review Board and all participants provided written informed consent.

Measurement of Butyrate Concentration in Stool

Concentrations of short chain fatty acids (SCFAs) such as butyrate were measured in the stool using gas chromatography/mass spectrometry (GCMS). Metabolites were extracted from samples using ice-cold methanol. Internal standards (n-methylserotonin and p-toluene sulfonic acid were added to each sample, vortexed, and incubated at -20C for 1 hour. Samples were then centrifuged and the supernatants drawn off and dried in a water bath under nitrogen gas. Samples were resuspended in water:acetonitrile, filtered, and stored. Extracts were then separated by reverse-phase HPLC on Zorbax C18 columns. The metabolites of interest were measured by tandem mass spectrometry on a triple quadrupole instrument. Metabolite peaks, identified by specific m/z transitions, were normalized to internal standards and concentrations calculated from standard curves processed and run in parallel with each batch. Levels of butyrate, acetate, and propionate were compared between the cohorts using the Mann-Whitney U Test (MWUT). Among patients with NSB, we correlated the butyrate level with the time to next NSB event using Pearson correlation.

Measurement of Abundance of Butyrate-producing Microbes in Stool

Stool samples were thawed to +4C, mixed with sterile saline, and bead beaten to physically lyse the samples. Microbial genomic DNA was isolated from stool using a MoBio PowerSoil kit. DNA concentration in each extract was measured and standardized. We measured the abundance of butyrate producing organisms in the microbiome by measuring expression of butyryl-CoA: acetate CoA-transferase (*but*) and butyrate kinase (*buk*) genes by

qPCR, normalized by sample mass. Measurement of both of these genes is essential to capture the majority of butyrate-producing microbes as many microbes express either *but* or *buk* or favor one over the other depending on ambient conditions. Expression of *but* and *buk* correlate with butyrate production.[127] We compared the difference in bleeders vs. non-bleeders using the Mann-Whitney U Test (MWUT).

Measurement of the Effect of LVADs on Endothelial Contraction

In a subset of patients, we measured the LVAD-related endothelial cell retraction using traction force microscopy (TFM).[128, 129] HUVECs were cultured on a 3D extracellular matrix embedded with fluorescent microspheres. Cells were then treated with plasma from patients with/without NSB. Cells were imaged on a confocal microscope at discrete time points and images were analyzed using ANSYS 8.0 software. We computed a continuous displacement field using these images. From this, we calculated the strain field and the stress field surrounding each cell using pre-determined information about the behavior of the hydrogel membrane. We then used the stress field to compute the traction force of each cell using normal vectors to the cell surface obtained from the 3D image stack. 20 cells were imaged for each patient and treatment condition. We used the Mann-Whitney U Test to compare the force between cohorts and the Wilcoxon Signed Rank Test to compare the change within cohorts between treatment conditions.

Results

Patient Enrollment

Forty-five adult patients with LVADs who were admitted to the University of Chicago Medical Center were enrolled. Clinical Characteristics are shown in Table 4.1. Three groups of patients with LVADs were studies: patients with no known history of GI bleeding (n=25), those who had bled in the past but were not currently bleeding (n=9), and those who were actively bleeding during their admission (n=11). Ten patients were supported with a Heartware HVAD, 23 with a Thoratec Heartmate 2, and 12 with a Thoratec Heartmate 3.

Butyrate inhibits TNF-α/Ang-2-induced angiogenesis

To investigate how butyrate affects TNF-α/Ang-2-induced angiogenesis, we incubated HUVECs on Matrigel with or without TNF-α/Ang-2 (12.5 ng/mL and 50 ng/mL, respectively)

Table 4.1 – Clinical Characteristics

	Never Bled	Bled in Past	Bleeding Now
Number of participants	25	9	11
Age (years)	55.1±11.8	62.4±12.4	59.6±12.7
Female (%)	24	33	36
Black race (%)	52	44	55
BMI (kg/m2)	31.6±7.5	28.7±7.1	30.2±9.0
eGFR (ml/min*1.73m^2)	68.1±31.1	73.4±29.9	60.4±31.7
Hypertension (%)	48	56	45
Diabetes mellitus (%)	44	44	72
Dyslipidemia (%)	20	22	27
Heart rate (beats/min)	84.7±12.3	93.8±20.7	92.7±12.9
Mean Arterial Pressure (mmHg)	79.1±10.1	78.6±16.5	79.8±11.0
Pulse Pressure (mmHg)	23.3±8.9	22.5±9.8	19.4±7.2
Hemoglobin (mg/dL)	10.8±1.9	10.4±2.6	8.9±1.1
B-type natriuretic peptide (ng/L)	4035.4±5597.7	4415.8±5762.5	14352±32798
Total cholesterol (mg/dL)	131.3±40.0	143.2±39.5	137.1±42.7
HDL (mg/dL)	39.5±18.7	47.0±24.0	44.5±20.1
LDL (mg/dL)	78.6±26.8	73.9±26.5	67.3±25.8
INR	2.2±0.6	2.1±0.5	1.9±0.5
Platelet count (#/μL)	217.8±47.4	211.9±35.2	187.1±69.9
C-Reactive Protein (mg/dL)	28.1±39.9	28.1±29.2	31.3±46.5
Lactate Dehydrogenase (U/L)	265.1±97.5	320.3±96.9	308.9±121.8

and with or without butyrate 2.5 mM. As shown in Figure 4.1, tubule formation was significantly higher in cultures treated with TNF-α/Ang-2 vs control (16.0±0.9 vs. 6.3±6.1 tubules per LPF respectively, and this effect was blunted by butyrate (7.6±2.5 tubules per LPF). No significant difference was seen in control cultures treated with butyrate (8.0±3.6 tubules per LPF).

Butyrate concentration is higher in the stool of LVAD patients with gastrointestinal bleeding

To determine whether LVAD patients with gastrointestinal bleeding had higher concentrations of butyrate in the stool, we collected stool samples from LVAD patients with and without GI bleeding and measured butyrate concentration by GCMS. As shown in Figure 4.2, stool butyrate concentration was 45% lower in LVAD patients with GI bleeding compared with non-bleeders (35.8±25.6 vs. 64.7±37.3 mM, respectively). Other fatty acids in the stool such as

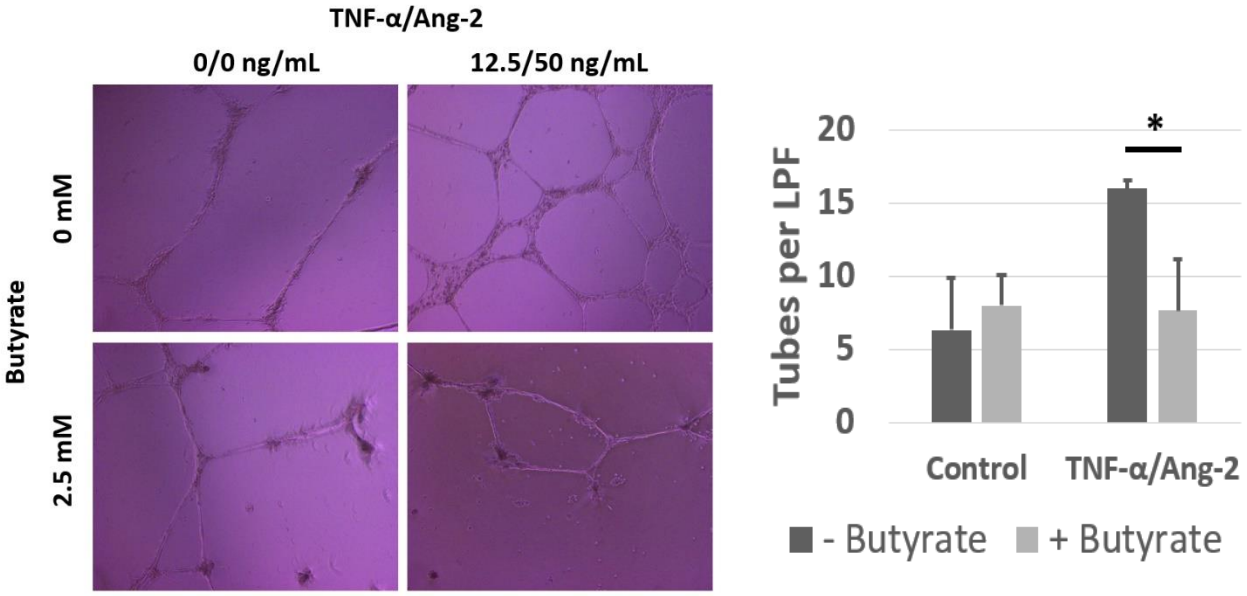


Figure 4.1: Butyrate inhibits TNF-α/Ang-2-induced angiogenesis. *HUVECs were incubated overnight on Matrigel with or without TNF-α/Ang-2 supplementation and/or sodium butyrate treatment. Tubule formation was measured by microscopy. TNF-α/Ang-2 together increased tube formation and this effect was blunted by butyrate. No significant effect was seen in cells treated with butyrate but without TNF-α/Ang-2.*

acetate and propionate were not significantly different between the groups.

Populations of butyrate-producing organisms are lower in the stool of LVAD patients with gastrointestinal bleeding

Because the butyrate concentration in the stool of LVAD patients with GI bleeding was lower than that of non-bleeders, we then hypothesized that the population of butyrate-producing organisms in the gut of the bleeders is also lower. To test this hypothesis, we isolated microbial genomic DNA from the stool of LVAD patients with and without GI bleeding and measured expression of acetate CoA-transferase (*but*) and butyrate kinase (*buk*) genes by qPCR, normalized by sample mass. As shown in Figure 4.3, the population of butyrate-producing organisms was substantially depleted in patients with a history of GI bleeding compared with those without any history of bleeding (0.25±0.41 vs. 0.61±0.76 AU, respectively). This difference was even larger between patients who were actively bleeding at the time of collection and those who had never bled (0.11±0.14 vs. 0.61±0.76 AU, respectively). Finally, the relative difference of

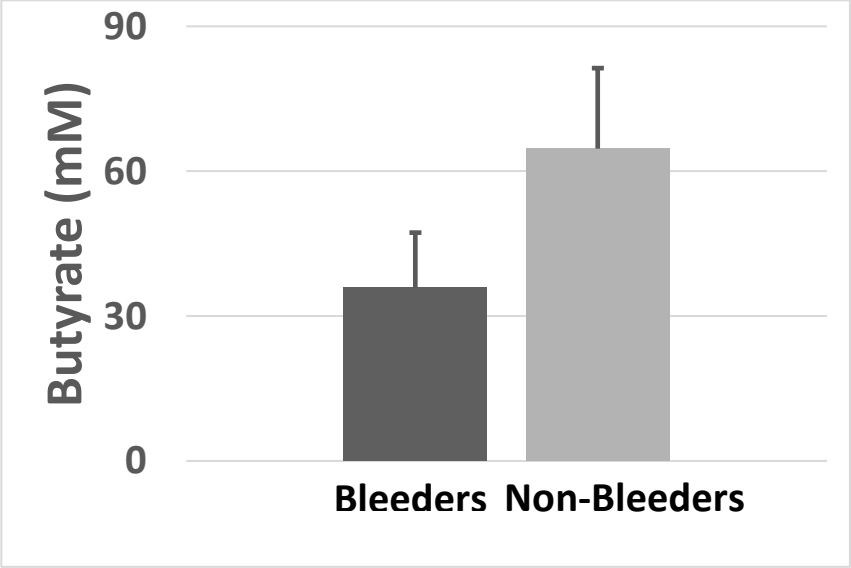


Figure 4.2: Butyrate concentration in the stool of LVAD patients with non-surgical bleeding is lower than in LVAD patients without non-surgical bleeding. *Stool was collected from hospitalized LVAD patients and butyrate concentration was measured by GCMS.*

butyrate-producing organisms in patients who were bleeding at the time of collection, those who had bled in the past but were not currently bleeding, and those who had never bled (0.11±0.14, 0.25±0.41, 0.61±0.76 AU, respectively), highlights the inverse relationship between butyrate-

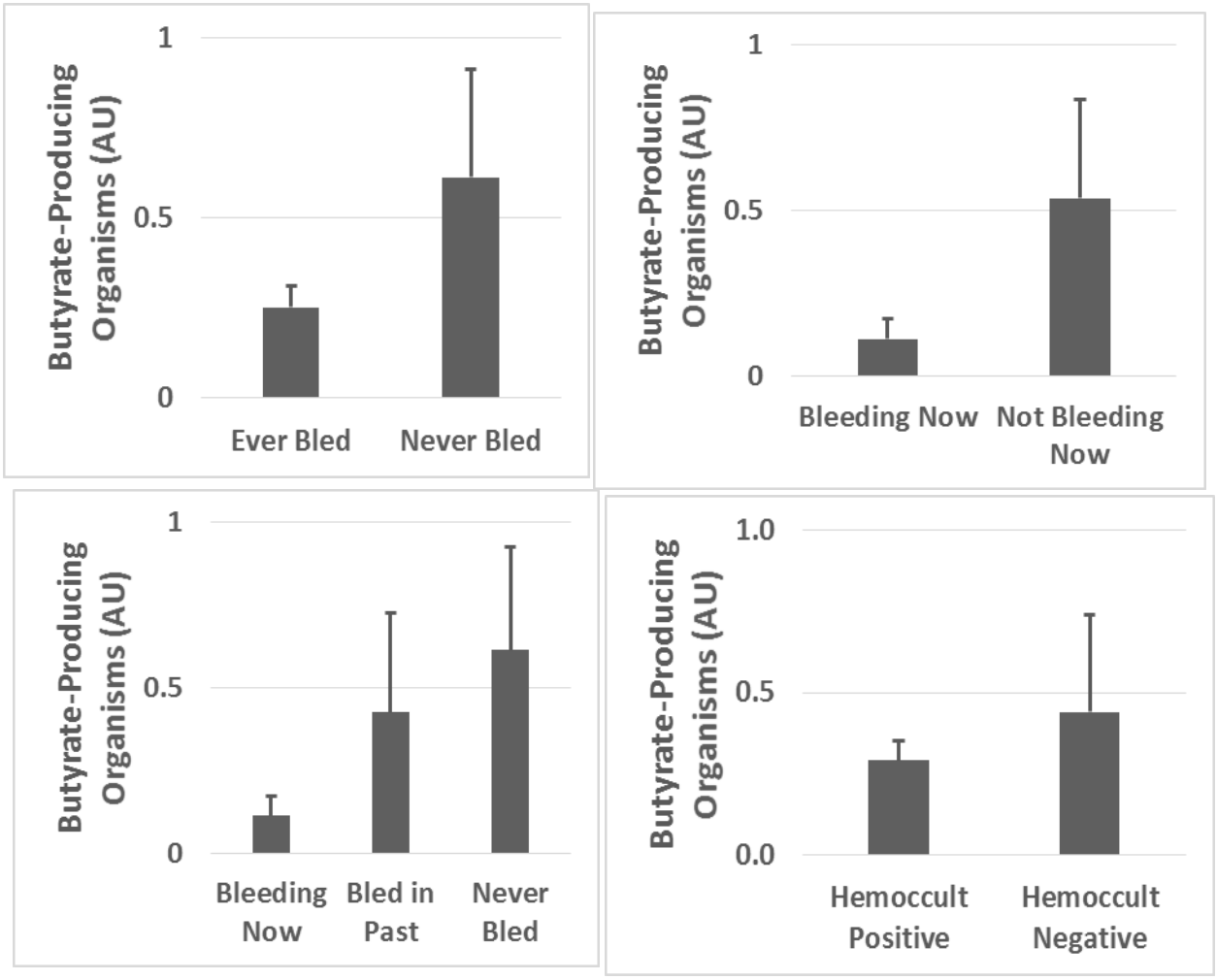


Figure 4.3: Butyrate-producing organisms are lower in LVAD patients with NSB. *Stool was collected from patients with LVADs with and without NSB and populations of butyrate-producing organisms were measured by qPCR. Patients with a history of bleeding had lower abundance of butyrate-producing organisms than patients who had never bled. Patients who were bleeding at the time of collection also had far lower abundance than those who were not bleeding at the time of collection. Finally, patients who were bleeding at the time of collection had the lowest abundance of butyrate-producing organisms while those who had never bled had the highest; those with a history of bleeding who were not actively bleeding at the time of collection were in the middle. Patients with a positive fecal occult blood test had slightly lower abundance of butyrate-producing organisms than those with a negative FOBT, however the use of iron supplementation in this population likely confounds this result.*

producing microbial population and propensity to bleed. Similarly, patients whose stool tested positive for blood had lower populations of butyrate-producing microbes in their stool than did patients whose stool was negative for blood (0.29±0.52 vs. 0.44±0.83 AU, respectively). However, this analysis may be confounded by the concomitant use of iron supplements which can affect the assay for blood in the stool. Unsurprisingly, the population of butyrate-producing microbes in the stool correlated directly with stool butyrate concentration (R=0.35).

Plasma from LVAD patients with NSB induces endothelial contraction

Because ROCK activity is higher in LVAD patients with NSB and ROCK is well-known to induce endothelial destabilization and permeability, we hypothesized that plasma from LVAD patients would induce endothelial contraction. To investigate this, we incubated HUVECs with plasma from patients with and without NSB and measured endothelial contraction force by traction force microscopy (TFM). Endothelial cell contraction force was significantly higher in cells treated with plasma from patients with NSB compared with those without NSB ($3.29x10^{-5}\pm1.46x10^{-5}$ vs $2.13x10^{-5}\pm1.42x10^{-5}$ pJ/μ^2, respectively, $p<0.05$). Both LVAD bleeders and non-bleeders had more endothelial contraction force than normal patients ($1.12x10^{-5}\pm7.8x10^{-6}$ pJ/μ^2), suggesting that even LVAD patients without NSB may have ongoing low levels of systemic inflammation.

Discussion

We have shown that gastrointestinal dysbiosis, specifically low levels of butyrate-producing microbes, is associated with gastrointestinal bleeding in human patients with continuous-flow LVADs. This dysbiosis is accompanied by low stool butyrate levels in LVAD patients with GI bleeding. We have further shown that butyrate inhibits angiogenesis stimulated by TNF-α and Ang-2 *in vitro* which we have previously shown are elevated in LVAD patients who bleed and drive LVAD-related angiodysplasia. We have also shown that plasma from LVAD patients who bleed induces endothelial cell contraction more so than plasma from LVAD patients with no history of bleeding. Endothelial cell contraction is mediated by Rho-kinase which we have previously shown to be activated in LVAD patients. Taken together, our findings suggest that gastrointestinal dysbiosis may be a key predisposing factor in the development of LVAD-related angiodysplasia.

Prior studies have described a link between the gastrointestinal microbiome and connective tissue diseases such as vasculitis.[130] Coit and colleagues described overabundance of *Haemophilus parainfluenzae* and depletion of *Alloprevotella rava* of the salivary microbiome in patients with Behcet's disease,[131] an inflammatory vasculitis, while Seoudi and colleagues reported overabundance of *Rothia denticarriosa*, *Streptococcus salivarius*, and *Streptococcus sanguinis* in Behcet's patients.[132] Consolandi and colleagues recently reported decreased butyrate levels and low levels of the butyrate-producer *Rusburia spp.* in the guts of Behcet's patients,[133] suggesting that low levels of anti-inflammatory butyrate could be a potential driver of the vascular lesions seen in Behcet's. Similarly, we have shown that loss of butyrate-producing gut microbes and low levels of butyrate in the gastrointestinal tract are associated with up-regulation of inflammatory and angiogenic pathways in LVAD patients who develop vascular pathology in the same tissue where the microbes are deficient. Our findings extend the work of

prior authors by describing possible mechanistic similarity between Behcet's disease, a known inflammatory vasculitis, and LVAD-related angiodysplasia where angiogenic and inflammatory factors are deranged. While further study is needed to determine whether dysbiosis causes LVAD-related angiodysplasia, our results suggest that the anti-inflammatory effects of butyrate treatment[134] might be a possible treatment for LVAD-related angiodysplasia and bleeding.

Prior authors have described angiogenic synergy between TNF-α and Ang-2.[119] Our findings extend this knowledge by demonstrating the butyrate treatment is sufficient to blunt the combined angiogenic effect of these factors. As we have previously shown that Ang-2[112] and TNF-α[126] are critical drivers of LVAD-related angiodysplasia, these findings importantly demonstrate that butyrate therapy may be an effective treatment to prevent or cure this pathology.

In this study, we have also shown that serum from LVAD patients with NSB induces endothelial contraction more so than serum from LVAD non-bleeders. Both angiogenesis[66, 67] and TNF-α-induced endothelial contraction[65] are mediated by ROCK and we have also shown that ROCK activity is elevated in LVAD patients.[126] While further study will be needed to determine if ROCK mediates the endothelial contraction induced by serum from LVAD patients with NSB, our findings are consistent with our proposed mechanistic model whereby inflammation leads to endothelial growth and permeability and ultimately leads to bleeding in butyrate-deficient LVAD patients.

Our study has several limitations. Stool samples were collected from hospitalized LVAD patients because stool collection at home (with subsequent immediate cold storage until analysis) would have been impractical. Changes in diet and location are known to alter the

microbiome. While we were careful to collect the first three stool samples from each patient to minimize the effects of the hospital diet on the microbial composition, we acknowledge that some degree of dietary confounding is likely. However, since all patients in the study were admitted to the same hospital, this effect would bias the results toward the null. Similarly, antibiotics and other supplements can alter the microbiome and we did not control for these in this pilot study. However, these agents are commonly used in the real-world LVAD population. The microbiome is known to vary throughout the gastrointestinal tract and stool analysis therefore does not allow specific microbiologic interrogation of specific enteric sites. While some authors recommend endoscopic evaluation of the microbiome through mucosal biopsy to mitigate this limitation, such a method would be unsafe in LVAD patients who are anticoagulated and coagulopathic. Therefore, as this limitation was inevitable, we chose to analyze stool in this study. Due to the danger of routine biopsies in these patients, we also were unable to measure endothelial contraction and permeability directly. Therefore, we developed an assay using traction force microscopy by exposing cultured cells to blood products from human patients. While we acknowledge this system does not fully mimic the *in vivo* condition, our results do suggest that constituents in the blood, likely inflammatory markers, induce endothelial contraction. Finally, we acknowledge the sample size is small in this pilot study, however this work was necessary to support a future confirmatory investigation which is ongoing.

Conclusions

Butyrate inhibits the angiogenic effects TNF-α and Ang-2 which drive LVAD-related angiodysplasia. LVAD patients with gastrointestinal bleeding have low levels of butyrate and butyrate-producing microbes in the gut. Therefore, gastrointestinal dysbiosis may explain why

some LVAD patients to develop bleeding and why the bleeding typically favors the gastrointestinal tract.

Chapter 5

Project Conclusions and Implications

Through these experiments, we have shown that high levels of Ang-2 and TNF-α in LVAD patients mediate angiodysplasia and vascular instability. These factors are critical mediators in the angiogenic and inflammatory systems respectively, both of which are activated in LVAD patients. Activation of these systems in LVAD patients induces pericyte apoptosis, altered angiogenesis, and endothelial expression of Tissue Factor, and is associated with Rho Kinase activation and endothelial contraction. While many factors likely contribute to activation of angiogenic and inflammatory cascades in LVAD patients, low levels of butyrate-producing

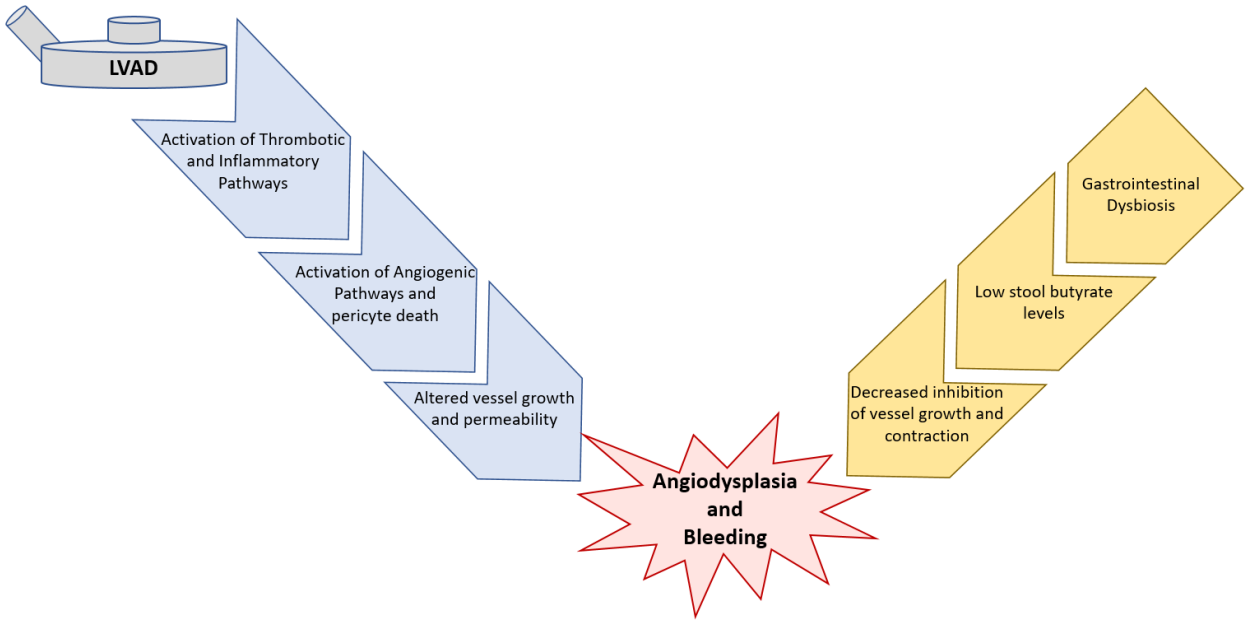


Figure 5.1: Proposed mechanism to explain LVAD-related gastrointestinal bleeding. *Interaction between the LVAD and the blood activates thrombotic and inflammatory pathways which in turn interact with vascular cells to activate angiogenic pathways and to kill pericytes, resulting in altered vessel growth and permeability. Patients with gastrointestinal dysbiosis, either pre-existing or iatrogenic, have low levels of stool butyrate and therefore lose butyrate's negative effect on gastrointestinal vessel growth and permeability. Thereby, vessel growth, contract, and leakage is left unchecked in the gastrointestinal tract which results in angiodysplasia and bleeding.*

microbes in the gastrointestinal tract is strongly associated with bleeding outcomes among these patients. While our findings suggest that Ang-2 and TNF-α are associated with NSB in LVAD patients, the GI tracts of these patients were not assessed directly in this study, and therefore it remains uncertain whether all of the LVAD-related bleeding in this study was due to angiodysplasia. Further study will be needed to determine whether pharmacologic inhibition of these pathways or manipulation of the gut microbiome can prevent or treat LVAD-related NSB. In particular, prospective *in vivo* studies in LVAD patients will need to be performed with Ang-2/TNF-α inhibitors to show cause and effect.

These studies describe for the first time a linear molecular mechanism to explain the observation of LVAD-related angiodysplasia and NSB (Figure 5.1). Durable forms of mechanical circulatory support such as LVADs represent the future of modern heart failure care and NSB remains the most common and costly complication of LVAD use. Therefore, understanding the molecular mechanisms leading to this troublesome outcome is critical to the more widespread use of LVADs and the future development of novel heart failure treatments. As small animal models of chronic mechanical circulatory support are currently impractical, and *in vitro* models of LVAD support fail to adequately mimic *in vivo* conditions, these studies are important both for the novel methods we designed and for the findings we reported which we and others will use to further our understanding of LVAD-related angiodysplasia.

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