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Superoxide and Pulmonary Hypertension

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SUPEROXIDE AND PULMONARY HYPERTENSION

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SUPEROXIDE AND PULMONARY HYPERTENSION

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Abstract: Pulmonary arterial hypertension (PAH) is a devastating cardiovascular disease, which is progressive and fatal despite advances in therapy. Animal models have shown that oxidative stress may be pathogenic in PAH. We hypothesize that oxidative stress, as measured by increased levels of a specific reactive oxygen species, superoxide ($O_2^{\bullet-}$), may play a role in PAH in humans. Using a novel application of electron paramagnetic resonance (EPR) spectroscopy, we initially evaluated whether levels of $O_2^{\bullet-}$ from whole blood collected from the pulmonary artery collected during right heart catheterization (RHC) would correlate with $O_2^{\bullet-}$ levels in peripheral venous blood. There was a significant correlation. We then measured $O_2^{\bullet-}$ in peripheral venous blood in patients with PAH and normal controls. We hypothesized that $O_2^{\bullet-}$ as a measure of oxidative stress would be elevated in patients with PAH compared with normal controls and that $O_2^{\bullet-}$ levels would correlate with measures of PAH severity. Results showed no significant elevation of $O_2^{\bullet-}$ levels in PAH patients versus controls. Furthermore, there were no correlations between levels of $O_2^{\bullet-}$ and severity of PAH. Contrary to previous animal studies and indirect measures of oxidative stress, we were unable to confirm an oxidative environment in PAH, at least when assessed by whole blood $O_2^{\bullet-}$ levels measured by EPR spectroscopy.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES:

LIST OF ABBREVIATIONS

AP-1	Activator Protein-1
ARE	Antioxidant Response Element
a.u.	Arbitrary Unit
BDI	Borg Dyspnea Index
BH_4	tetrahydrobiopterin
BMPR	Bone Morphogenetic Protein Receptor
BNP	B-type natriuretic peptide
Ca^{2+}	Calcium
cGMP	cyclic Guanosine MonoPhosphate
CI	Cardiac Index
CO	Cardiac Output
CTD	Connective Tissue Disease
CTEPH	Chronic Thromboembolic Pulmonary Hypertension
DLCO	Diffusion capacity of the Lung for Carbon Monoxide
DNA	Deoxyribonucleic acid

eNOS	endothelial Nitric Oxide Synthase
EPR	Electron Paramagnetic Resonance
ET	Endothelin
FHR	Fawn Hooded Rats
H_20_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible transcription factor-alpha
IPAH	Idiopathic Pulmonary Arterial Hypertension
Keap-1	kelch-like ECH- associated protein 1
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
MCT	Monocrotaline
MDA	Malondialdehyde
MVO_2 sats	Mixed venous oxygen saturations
6MW	Six minute walk
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFκB	Nuclear Factor kappa-light chain-enhancer of activated B cells
NO•	Nitric Oxide
NOS	Nitric Oxide Synthase
NOX	Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase
Nrf2	nuclear factor erythroid 2-related factor 2
OH•	Hydroxyl radical
$O_2^{\bullet-}$	Superoxide
PA	Pulmonary Artery
PAC	Pulmonary Artery Capacitance
PAEC	Pulmonary Artery Endothelial Cells
PAH	Pulmonary Arterial Hypertension

PAOP	Pulmonary Artery Occlusion Pressure
PAP	Pulmonary Artery Pressure
PASMC	Pulmonary Artery Smooth Muscle Cells
PASP	Pulmonary Artery Systolic Pressure
PCOOH	Phosphatidylcholine hydroperoxide
PH	Pulmonary Hypertension
PPARγ	Peroxisome Proliferator Activated Receptor gamma
PVR	Pulmonary Vascular Resistance
RA	Right Atrium
RAP	Right Atrial Pressure
RHC	Right Heart Catheterization
ROCK	Rho kinases
ROS	Reactive Oxygen Species
RV	Right Ventricle
RVH	Right Ventricular Hypertrophy
RVSP	Right Ventricular Systolic Pressure
SiRNA	small interfering ribonucleic acid
SIRT	Sirtuin
SOD	Superoxide Dismutase
TAPSE	Tricuspid Annular Plane Systolic Excursion
SSc	Systemic sclerosis or scleroderma
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WU	Wood Unit
XO	Xanthine Oxidase

CHAPTER 1 INTRODUCTION:

PULMONARY HYPERTENSION

Pulmonary hypertension (PH) is a cardiovascular disease that results in significant limitations in functional capacity, reduced quality of life, and markedly decreased life expectancy. PH is a pathophysiological disorder that is defined by an increase in mean pulmonary arterial pressure (PAP) as assessed by right heart catheterization (RHC). The World Health Organization (WHO) has classified PH into 5 groups based on etiology. Group 1 is pulmonary arterial hypertension (PAH) a disease of the small pulmonary arteries characterized by vascular proliferation and remodeling.[1] Group 2 is the most common cause of PH, known as pulmonary venous hypertension due to left heart failure. In Group 2, PH is initially due to passive venous congestion from systolic or diastolic dysfunction or valvular disease. Later, Group 2 PH may develop arteriopathy due to chronic vasoconstriction with associated vascular remodeling. Group 3 is PAH due to chronic hypoxemic lung disease with hypoxic vasoconstriction leading to PAH. Group 4 is PAH due to unresolved thromboembolism, called chronic thromboembolic pulmonary hypertension (CTEPH). Group 5 is PH due to miscellaneous systemic diseases. Current treatment guidelines recommend specific therapeutic PH medications only for WHO Group 1 and Group 4 PAH, which are the focus of this study.[2,3]

Normal pulmonary arterial pressures (PAP) are mean PAP 12-14 mm Hg with a pulmonary vascular resistance (PVR) less than 1.5 Wood units (WU). PAH is a progressive, life-threatening disease defined by RHC as an increase in mean PAP greater than 20 mm Hg (2 standard deviations above normal), PVR greater than 3 WU, and a pulmonary artery occlusion pressure (PAOP) less than 15 mm Hg.[3] A PAOP greater than 15 mm Hg is consistent with Group 2, pulmonary venous hypertension, wherein the high

PAP originates from elevations in the left atrial pressure, not from disease of the pulmonary arterioles.

Group 1 PAH consists of a variety of disorders with similar pulmonary vascular pathophysiological mechanisms. Group 1 PAH can be idiopathic (iPAH), hereditable, or associated with medications. Group 1 PAH may also be associated with other conditions including connective tissue diseases (CTD), especially systemic sclerosis (SSc), cirrhosis, infections such as HIV and schistosomiasis, and congenital heart disease.

Current treatments for PAH target three pathways important in endothelial function: 1) prostacyclin and 2) nitric oxide (NO•) which are under-expressed in PAH patients; and 3) endothelin (ET), which is overexpressed in PAH patients. The first pathway identified in PAH was the prostaglandin pathway.[4] Patients with PAH have decreased levels of prostaglandin I2, or prostacyclin, which is an eicosanoid that functions as a potent vasodilator and is responsible for increased production of thromboxane A2 and platelet aggregation. The second pathway that is targeted by current medical therapy is the NO• pathway. Decreased levels of NO•, a reactive nitrogen species and a potent vasodilator and antiproliferative molecule, and cyclic guanosine monophosphate (cGMP) have been described in PAH.[5] The third pathway currently targeted by medication is endothelin-1 (ET-1), which is a potent vasoconstrictor and mitogen in smooth muscle cells.[6] Medications targeting these pathways have changed the landscape of treatment for PAH over the past two decades, with significant improvement in symptoms and mortality. However, despite these treatments, patients still often progress to right heart failure and premature death. Prognosis remains poor with a mortality of 10-15% per year and median survival of approximately seven years.[7] Thus, new treatment pathways are needed to improve outcomes for patients with PAH.

Severity of disease in PAH is assessed clinically using the World Health Organization (WHO) functional class.[8] Functional class 1 patients do not have significant

dyspnea. Functional class 2 patients have dyspnea with strenuous activity. Functional class 3 patients have dyspnea with minimal activity. Functional class 4 patients have dyspnea at rest. The REVEAL risk score is also used to assess severity and estimate prognosis in patients with PAH.[9] In addition to WHO functional class, the REVEAL risk score includes demographics, comorbidities, vital signs, hospitalizations, six minute walk (6MW) test distance, B-type natriuretic peptide levels (BNP), the diffusing capacity for carbon monoxide (DLCO) on pulmonary function testing, and findings on echocardiography and RHC.

The pathogenesis of WHO Group 1 PAH is a complex multifactorial process wherein the increases in PVR and mean PAP are the result of pulmonary vascular remodeling.[1] This includes muscularization of peripheral arteries, medial hypertrophy of muscular arteries, loss of small pre-capillary arteries, neointima formation, and eventually endothelial cell proliferation. In the later stages of the disease, endothelial cell proliferation leads to the development of plexiform lesions which are aberrant channels in the obliterated vessel lumen and in the adventitia. These lasting alterations in vessel structure contribute to an increase in PAP and PVR. The stimuli responsible for remodeling involve changes in transmural pressure, stretch, shear stress, hypoxia, various mediators such as ET, and increased levels of reactive oxygen species (ROS).

Group 4 PAH, CTEPH, develops when clot in the pulmonary arteries does not resolve and transforms into fibrous tissue with dysfunction and remodeling of the pulmonary microvasculature similar to the arteriopathy of PAH. Pulmonary endarterectomy is the reference-standard treatment, but some patients are ineligible for surgery or have persistent PAH following surgery or balloon angioplasty.

Current medical treatments of Group 1 and Group 4 PAH target three pathways: NO•, ET, and prostacyclin. However, mortality remains high and further therapies are

needed. In addition to imbalances in NO•, prostacyclin, and ET, evidence suggests that ROS contribute to the pathogenesis of Group 1 PAH and CTEPH.[10,11]

REACTIVE OXYGEN SPECIES

Current evidence suggests that ROS contribute to PH pathogenesis by activating inflammatory signaling, altering vascular cell proliferation and apoptosis resistance, and inducing hypertrophy of pulmonary arterial smooth muscle cells (PASMC). ROS were first described by Fenton in 1894 and they include free-radical species such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical (OH•) and non-radical oxidants such as hydrogen peroxide (H_2O_2). ROS can be formed non-enzymatically in reactions catalyzed by metals. In biology, ROS were initially recognized as unwanted byproducts of oxidative phosphorylation in mitochondria. Electrically charged $O_2^{\bullet-}$ is highly reactive and is rapidly converted to H_2O_2 by the superoxide dismutase (SOD) family of enzymes, including manganese- and copper/zinc-SOD. In the 1990s, ROS, including $O_2^{\bullet-}$, became recognized as important signaling molecules in a variety of physiological and pathophysiological conditions. Superoxide is considered to be an intra-cellular messenger due to the low cellular concentrations of $O_2^{\bullet-}$ and its limited diffusion, whereas H_2O_2 is both an intra- and inter-cellular messenger.

ROS modulate cell adhesion and migration, growth and proliferation,[12,13] and resistance to apoptosis of the endothelium.[10] $O_2^{\bullet-}$ can directly stimulate PASMC proliferation.[14] Hypoxia-induced ROS generation in PASMC contributes to the expression and activity of the hypoxia-inducible factors (HIF-1α and HIF2α) involved in PASMC proliferation.[15] Additionally, $O_2^{\bullet-}$ directly triggers Calcium (Ca^{2+}) sensitization and vasoconstriction in rat pulmonary arteries.[16,17]

ROS can also influence a broad spectrum of physiological processes through the modification of enzymes, transcription factors, and other metabolic intermediates. The

modification of transcription factors by interaction with ROS can affect their translocation and binding to elements/promoters in the genome, representing an important redox-sensitive function. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a good example of a transcription factor that is ROS sensitive.[18] In normal conditions, Nrf2 is bound to the kelch-like ECH-associated protein 1 (Keap1)-nuclear factor and remains inactive. An increase in intracellular ROS leads to oxidative modification of key cysteine residues on Keap1, resulting in Nrf2/Keap1 dissociation. Upon activation, Nrf2 is translocated to the nucleus and binds to the antioxidant response element (ARE), also termed the electrophilic response element, which is a "master" transcription regulator of many antioxidant enzymatic systems.[19] The Keap1–Nrf2–ARE pathway is thus activated under increased oxidative stress to restore the redox equilibrium, by promoting transcription of response genes, and to induce anti-inflammatory and antioxidant responses. Activator protein-1 (AP-1) and nuclear factor κB (NFκB), two other redox-sensitive transcription factors, are upregulated by ROS that are generated subsequent to acute blood flow cessation.[20] Additionally, the p50 subunit of NFκB requires cysteine oxidation to bind DNA as the basis for its redox sensitivity.[21] Both NFκB and AP-1 have the potential to drive a proinflammatory phenotype in the endothelium through the upregulation of adhesion molecules such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin. Further, in experimental PH, ROS increase the expression and/or activation of multiple factors involved in cellular growth.[22-24]

ROS can also stimulate both the expression of the ET-1 gene and the secretion of the ET-1 peptide.[25] Endothelin is a potent vasoconstrictor and levels are elevated in PAH, with current medical therapy directed at antagonizing the ET receptor.

In the lungs, endothelial cells, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells are all sites of ROS generation. ROS in the pulmonary vasculature can be produced from complexes in the cell membrane, cellular organelles such as

peroxisomes and mitochondria, and in the cytoplasm. In addition, low levels of substrates of co-factors such as L-arginine and tetrahydrobiopterin (BH_4) can uncouple nitric oxide synthase (NOS), producing a shift from NO• generation to ROS production, specifically $O_2^{\bullet-}$. Several enzymes are recognized to be involved in generating the increased $O_2^{\bullet-}$ associated with PAH.[26]

NADPH OXIDASE (NOX)

The Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases (NOX) comprise a family of 7 structurally related transmembrane NADPH dependent oxidoreductases that include the NOX enzymes and the Duoxes. All of the NOX enzymes share the same basic organizational scheme consisting of 6 transmembrane domains that support two oxygen binding heme moieties, a C-terminal Flavin and NADPH binding domain. This results in the transfer of $O_2^{\bullet-}$ to the opposite side of the membrane. NOX enzymes are located on the cell membrane and other organelles of polymorphonuclear leukocytes, macrophages, PASMCs, fibroblasts, and pulmonary artery endothelial cells (PAECs). NOXs generate $O_2^{\bullet-}$ by transferring electrons from NADPH via a catalytic core protein to molecular oxygen. The NOX family comprises 5 isoforms that display distinct patterns of tissue specifically. NOX2 was the first recognized in its role in innate immune response in phagocytes by generating $O_2^{\bullet-}$ in the respiratory burst.[27]

NOXs have been recognized as important sources of ROS in the vasculature. In the cardiovascular system, NOX-1, NOX-2, NOX-4 and NOX-5 have been detected.[28] NOX-1 and NOX-2 require other subunits for activation and produce $O_2^{\bullet-}$. NOX-4 acts constitutively and produces H_2O_2. NOX-5 has only been found in humans and is the only NOX isoform that can be directly activated by Ca^{2+}.[29] Vascular smooth muscle cells and endothelial cells express NOX-1, NOX-2, NOX-4, and NOX-5.[30,31]

Initial models that identified the role of NOX evaluated WHO group 3 PH, which is due to pulmonary disease with hypoxic vasoconstriction. Increased expression of NOX-1 and NOX-2 is seen in the monocrotaline rat model of PH.[32] Lung tissue specimens from PAH and non-PAH patients have shown an upregulation of NOX-1 at the transcript and protein level in resistance vessels in lung specimens from PAH patients compared with non-PAH tissue samples.[33] This upregulation of NOX-1 coincided with an increase in ROS production as measured by cytochrome c and Amplex red assays and an increase in the expression of bone morphogenetic protein receptor (BMPR) antagonist Gremlin 1. BMPR type II receptor mutations underlie most cases of heritable PAH.[34] In hypoxia-exposed human PAECs, NOX-1 subunit expression was induced resulting in elevation in Gremlin 1 expression. NOX-1 gene-silencing abrogated this cascade. Loss of NOX-1 or Gremlin 1 attenuated hypoxia-induced endothelial cell proliferation. Similarly, in another study, NOX-1 promoted BMPR antagonist Gremlin 1-driven PAEC proliferation and PAH.[35]

NOX-2 has also been shown to be important in the development of PH using NOX-2 knockout mice models with hypoxia.[36,37] Chronic hypoxia increased $O_2^{\bullet-}$ production in a process dependent on NOX-2 and resulted in vascular remodeling and enhanced vasoreactivity. Disruption by gene knockout of NOX-2 completely abolished PH and vascular remodeling and attenuated $O_2^{\bullet-}$ production.

Enhanced NOX-4 levels have been identified in PASMCs exposed to hypoxia.[12,38] Hypoxia-inducible transcription factors (HIFs) have been recognized as master regulators of oxygen-regulated gene expression. HIF-1 is the most abundant member. It is a heterodimer that has been implicated in physiological and pathophysiological responses to hypoxia.[39] In PASMCs from mice exposed to hypoxia, HIF-1α overexpression has been shown to enhance NOX-4 mRNA and protein levels.[40] Elevated expression of NOX-4 was detected in monocrotaline rats with PH and in human fibroblasts, PAECs and PASMCs by real-time PCR. NOX-4 inhibitors were effective at halting progression of PH in the rats.[41]

In mice with PH due to pulmonary artery (PA) banding, there was upregulation of NOX-4 mRNA and protein within hours with a subsequent increase in NOX-2 mRNA.[42]

NOX-5 is expressed in endothelial cells in the media and fibroblasts in the adventitia of human pulmonary blood vessels.[43]

In summary, NADPH oxidases are likely the principal source of $O_2^{\bullet-}$ in the vasculature in PH. In addition, NOXes also regulate the activities of other ROS-generating oxidases such as Xanthine Oxidase (XO) and NOS.

UNCOUPLED NITRIC OXIDE SYNTHASE (NOS)

Another species critical in redox balance is NO•, which is a potent vasodilator, neurotransmitter, second messenger, inflammatory marker and therapeutic agent in PAH. NO• is the principal mediator of flow-mediated dilation and is responsible for the inhibition of platelet aggregation, leukocyte adhesion, and smooth muscle proliferation. NO• is formed during the conversion of the amino acid L-arginine to L-citrulline by a specific enzyme, NOS. By interacting with the heme moiety of the enzyme soluble guanylate cyclase, NO• stimulates the production of cGMP. The elevation of intracellular cGMP results in activation of Ca^{2+}-activated potassium channels, membrane hyperpolarization and relaxation of vascular tone. Three NOS isoforms have been identified. Endothelial NOS (eNOS) is found in the endothelial lining of blood vessels. NOS is a homodimer that requires the cofactor BH_4. Zinc stabilizes the dimer. Under some conditions, eNOS generates $O_2^{\bullet-}$ rather than NO•, a phenomenon known as NOS uncoupling. Decreased availability of BH_4, oxidation of zinc, and L-arginine depletion can lead to NOS uncoupling.[29] An oxidized cellular environment also induces NOS uncoupling so that it generates $O_2^{\bullet-}$ instead of NO•. When $O_2^{\bullet-}$ production is increased, it can rapidly interact with NO• and attenuate its capacity to stimulate soluble guanylate cyclase,[44] which leads to less vasodilation of the pulmonary vasculature. The equipoise between ROS and RNS

regulates several intracellular pathways. In conditions that lead to an imbalance between enhanced ROS production and reduced antioxidant response, the excess $O_2^{\bullet-}$ reacts with NO•, reducing its bioavailability and producing peroxynitrite. Peroxynitrite is a potent oxidant which causes nitration of many molecules, altering their function and structure.

DYSFUNCTIONAL MITOCHONDRIA

Mitochondria underlie the unique ability of the pulmonary circulation to vasoconstrict with hypoxia. Mitochondria respond to reduced PO_2 by altering the production of ROS which compromises potassium channel function. This leads to the depolarization of PASMCs, activation of voltage sensitive Ca^{2+} channels, and smooth muscle contraction.[28] ROS are normally produced as byproducts of aerobic metabolism by the electron transport complexes, with 1-2% of oxygen being converted to $O_2^{\bullet-}$. Tricarboxylic acid cycle and electron transport chain modulations are associated with alterations in ROS.[45] Superoxide can be generated by complex I or complex III. Similar to metabolic reprogramming that is a hallmark of cancer, incomplete mitochondrial metabolism with the accumulation of carboxylic acid intermediates has been identified in PASMCs, PAECs and fibroblasts.[46] Abnormalities in mitochondrial function have been identified in PH including reduced expression of components of the mitochondrial electron transport, reduced SOD2 expression levels, changes in mitochondrial membrane potential, altered oxygen sensing, and a shift to glycolysis.[47-49] In PH, acquired mitochondrial abnormalities, including epigenetic silencing of SOD2,[47] disrupt oxygen sensing with normoxic activation of HIF-1α and a metabolic shift to aerobic glycolysis. Mitochondrial ROS interact with NOXs and other ROS sources to collectively induce a pro-oxidative redox state, which is further favored by impairment of the antioxidant capacity of PH cells.[50,51] The redox-sensitive Nrf2 is a transcription factor that decreases

ROS generation. In preclinical PH studies, Nrf2 activation improves mitochondrial dysregulation, decreases ROS and consequently improves arterial and right ventricular (RV) remodeling.[52] Sirtuin 3 (SIRT) protein, the predominant NAD+-dependent mitochondrial deacetylase, can regulate mitochondrial ROS production. Human iPAH has been associated with a loss-of-function SIRT3 polymorphism.[53] Knockdown of SIRT3 in mice promotes spontaneous development of PH.[53]

OTHER SOURCES OF ROS

Xanthine oxidase (XO), the final enzyme of purine catabolism, has been identified in the PA endothelium in vitro and in vivo.[44] XO is a potent generator of ROS that has been implicated in the development of PH. XO expression and activity is increased in hypoxia and inhibition of enzyme activity with allopurinol blunts hypoxia-induced vascular remodeling.[54,55] XO can be activated by hypoxia in cultured PAECs. In rats exposed to hypoxia, XO activity is elevated.[54] In humans, uric acid is an independent risk factor for mortality in PH.[56]

Other enzymatic and nonenzymatic sources of ROS may be pathogenic in PH including lipoxygenase, cytochrome P450, cyclooxygenase, monoamine oxidase, iron, quinones. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a scavenger receptor that initiates $O_2^{\bullet-}$ production via NOX and up-regulates the redox-sensitive NF-κB and has been implicated in pulmonary arterial remodeling in PH animals.[57] LOX-1 is upregulated in a rat model of hypoxia-induced right ventricular hypertrophy (RVH).[58]

ANTIOXIDANTS

Normally, ROS production is counterbalanced by cellular antioxidant systems. ROS scavenging systems include SOD, catalase, and glutathione peroxidase. SODs convert $O_2^{\bullet-}$ to H_20_2. SOD has three isoforms: cytoplasmic (SOD1), mitochondrial (SOD2)

and extracellular (SOD3). All 3 isoforms of SOD are expressed in the lung but most of the studies linking SOD to PH involve SOD2. SOD2 expression in PASMC is decreased in PAH patients and fawn-hooded rats (FHR) with PAH. Administration of SOD2 small interfering ribonucleic acid (siRNA) to normal PASMC recapitulates a PAH phenotype.[50] SOD2 over-expression or therapy with SOD-mimetic MnTBAP reverses PAH. Epigenetic SOD2 deficiency impairs redox signaling and creates a proliferative, apoptosis-resistant PASMC.[50] Polymorphisms in the SOD2 gene can increase the risk of PAH.[59]

Heme oxygenase is an antioxidant enzyme that has been implicated in PH. Induction of heme oxygenase prevents the development of PH.[60] In the absence of heme oxygenase, cardiomyocytes have a maladaptive response to hypoxia and subsequent PH.[61] Other antioxidants that may be important in the pathogenesis of PH include catalase, which converts H_20_2 to water. Peroxiredoxins, glutaredoxin and thioredoxin may also play a role. Nonenzymatic antioxidant systems include vitamins C and E, retinol, glutathione and B-carotene.[62]

OXIDATIVE STRESS

Oxidative stress is the production of oxidizing species that exceed the capacity of enzymatic and chemical antioxidant defenses and cause a deviation from homeostasis.[63] When there is an imbalance between ROS production and ROS metabolism leading to increased ROS levels, this is often referred to as oxidative stress. This imbalance in ROS has been shown in animal models of PH with modulation of the release of ET-1 and prostacyclin. NOX derived $O_2^{\cdot-}$ has been associated with a decrease in prostacyclin in monocrotaline-induced PH.[64] Oxidative stress can stimulate both the expression of the ET-1 gene and the secretion of the ET-1 peptide.[25] ROS also modulate the release of peroxisome proliferator activated receptor gamma (PPARγ), which can acutely influence vessel tone and eventually result in vascular remodeling by modulating growth and

proliferation of PAECs. Chronic hypoxia-induced increases in NOX-4 were associated with reductions in the expression of PPARγ in human PASMC and PAECs.[65] Oxidative stress can also directly promote SMC contraction by triggering a rise in cytosolic free Ca^{2+} concentration. ROS increase the expression and/or activation of multiple factors involved in cellular growth.

Oxidant injury refers to the end result of oxidative macro-molecule modifications such as lipid peroxidation, oxidatively modified nucleotides, and protein and carbohydrate oxidation.[63,66] Dysregulation of ROS signaling and the subsequent oxidation of cellular protein, lipid and DNA is a major molecular switch which initiates and perpetuates vascular disease states.[67] Redox-sensitive signaling is crucial to control gene expression of angiogenic proteins such as vascular endothelial growth factor (VEGF), epidermal growth factor receptor, and platelet derived growth factor.[68] Redox signaling results in numerous effector responses including cellular differentiation, proliferation, migration, apoptosis, and antioxidant gene expression. Some of the best studied signaling effectors include the mitogen-activated protein kinases, protein tyrosine phosphatases, NF-κB, AP-1 and the Nrf2/keap pathways.[67] One of the major molecular targets of ROS is lipid peroxidation which results in the generation of isoprostanes and malondialdehyde (MDA). Isoprostane can bind to a prostanoid receptor and trigger downstream signaling to increase ET liberation, vascular tone, and smooth muscle proliferation. Isoprostanes have been shown to be increased in the urine of patients with PAH and correlate with poor survival.[69]

METHODS FOR ASSESSING OXIDATIVE STRESS

Direct measurements of ROS production are very difficult due to their high reactivity and low steady-state concentration.[70] Many groups have assessed oxidative stress via nonspecific methods. There are a variety of dyes that become fluorescent upon reaction with particular ROS. They are easy and relatively inexpensive, but lack of

specificity and true quantitation are significant drawbacks. Many methods are available for quantifying the products of ROS interactions with biological macromolecules. Lipid peroxidation products can be quantified by mass spectrometry, the thiobarbituric acid reactive substances assay, enzyme-linked immunosorbent assay, high-performance liquid chromatography and various colorimetric assays. Nucleotide and protein oxidation can be quantified by many of the same methods.

Our group and others have utilized Electron Paramagnetic Resonance (EPR) Spectroscopy, considered by many to be the reference-standard for detecting free radicals, [70,71] to quantify levels of the specific ROS, $O_2^{\bullet -}$. EPR Spectroscopy is quantitative and specific and can be used with biological samples, but the technique requires a great deal of technical expertise and specialized equipment. EPR Spectroscopy measures unpaired electrons. The unpaired electron will resonate at a different magnetic field strength according to the type of radical it occupies.[72] The spin probes, cyclic hydroxylamines rapidly react with $O_2^{\bullet -}$ to undergo oxidation to stable nitroxide, which can be measured by EPR. Cyclic hydroxylamines provide quantitative measurements of $O_2^{\bullet -}$ with high sensitivity.[71] Superoxide levels have been found to be elevated by EPR in whole blood samples from workers with occupational exposure to low levels of ionizing radiation compared to normal controls.[73] Superoxide was evaluated by EPR in six patients with congestive heart failure and found that treatment with statins reduced levels of $O_2^{\bullet -}$.[74] Superoxide measurement by EPR has not been evaluated in patients with PH.

ANIMAL MODELS OF PH AND OXIDATIVE STRESS

Studies in animals have suggested that an unconstrained elevation in ROS affects ion channel function and potentiates depolarization-induced Ca^{2+} entry, contributing to the enhanced contractile and remodeling processes that are characteristic of PH. There are multiple animal models of pulmonary hypertension. Some of the most common animal

models include hypoxic mice and rats often in association with monocrotaline (MCT) or Sugen, FHR, and caveolin-1 knock out mice. There are also PH models associated with shunting in neonatal lambs and in hypoxic piglets.

One of the most used models of PH is the MCT rat, established in 1967. MCT is a pyrrolizidine alkaloid which selectively injures the pulmonary vascular endothelium. Rats treated with MCT develop progressive pulmonary arterial structural changes resulting in PH and RVH progressing to right heart failure within weeks. MCT treatment induces a state of oxidative stress as indicated by elevated levels of $O_2^{\bullet-}$ in the pulmonary arteries,[75] and a significant increase in lipid peroxidation and a decrease in glutathione content in lung and RV tissues. Monocrotaline rats have increased expression of NOX-2 and NOX-4 and a decrease in catalase. BMPR1 and BMPR2 are significantly reduced.[24] Another model of PH uses rats injected with the VEGF-receptor inhibitor SU5416 or Sugen, often combined with hypoxia. Treatment with the VEGF receptor blocker induces endothelial cell proliferation leading to progressive vasculopathy with plexiform lesions similar to the pathology seen in human PAH. Obliterative vascular remodeling causes severe irreversible PH associated with precapillary arterial endothelial proliferation in chronically hypoxic rats.[76]

Male transgenic Ren2 rats have been used to show that Renin-angiotensin overexpression and oxidative stress contribute to PH and pulmonary vascular remodeling.[23] In these Ren2 rats, right ventricular systolic pressure (RVSP), NOX and ROS were elevated.[23]

In chronic hypoxia-induced PH mice, an increase in ROS, evaluated using dihydroethidium staining, was seen in pulmonary arteries.[36] In a rat model of PH induced by hypoxia, oxidative stress was assessed by lung tissue levels of xanthine oxidase and phosphatidylcholine hydroperoxide (PCOOH), a primary peroxidation product of phosphatidyl choline. Both XO and PCOOH were elevated in rats exposed to hypoxia.[54]

A strain of FHR develops PAH spontaneously. FHR-PAH is associated with increased ET levels, enhanced vasoconstriction and excessive PASMC proliferation.[47] Expression of SOD2 was decreased in FHR PASMCs.[47]

In a shunt lamb model of PH, $O_2^{\bullet-}$ and H_2O_2, as measured by oxidant-sensitive fluorogenic probes, were found to be elevated due to increased activity of NOX and uncoupled eNOS.[77] In another model, a Gore-Tex graft is anastomosed between the ascending aorta and main PA in anesthetized late-gestation lambs. They showed evidence of altered glucose catabolism which was accompanied by an increase in NOX expression and $O_2^{\bullet-}$ production.[13]

TREATMENT OF OXIDATIVE STRESS IN PH

Treatment of oxidative stress in animal models of PH has resulted in promising results. The antioxidant, diosgenin, attenuated progression of PH in monocrotaline rats by scavenging free radicals and increasing GSH content.[22] In MCT rats, treatment with bone marrow derived mesenchymal stem cells transduced with lentiviral vectors expressing eNOS decreased RVSP and improved survival. Alpha-smooth muscle actin was decreased while eNOS and serum NO• concentration was increased.[78]

Resveratrol (3,5,4'-trihydroxystilbene) is a dietary polyphenolic compound which exerts anti-proliferative, anti-oxidant, and anti-inflammatory effects in the pulmonary circulation of MCT-treated rats.[32] RVSP was normalized, RVH was prevented, and PAEC proliferation was attenuated by resveratrol. Further, it significantly decreased oxidative stress, detected with dihydroethidine, with down-regulation of NOX-1 and improved eNOS expression.

In an innovative study evaluating the effects of antioxidant treatments, SOD and fasudil, a specific rho-kinase inhibitor, were combined into a liposomal formulation in MCT-induced PH rats. This elicited a prolonged reduction in mPAP showing that antioxidant

mechanisms can be used to attenuate PH in rats.[79] A different PH model involving male REN2 rats showed that treatment with Tempol, a SOD mimetic that directly scavenges $O_2^{\bullet}$, decreased intrapulmonary NOX and ROS and RVSP.[23]

In a hypoxic rat model, treatment with the antioxidant N-acetylcysteine and the xanthine oxidase inhibitor allopurinol, inhibited the increase in PCOOH levels and reduced PH, RVH, and pulmonary vascular media thickening.[54] In another study using rats with PH induced by hypoxia, the SOD mimetic, tempol, prevented a rise in RVSP and increased RV contractility.[80] Tempol also prevented the hypoxia-induced rise in serum MDA levels. In neonatal lambs with persistent PH, SOD restored eNOS expression and function in resistance pulmonary arteries.[81]

In rats, hypoxia with SU5416 exposure caused severe PH with RVH, and greater vasoconstrictor sensitivity through a $O_2^{\bullet-}$ and Rho kinase-dependent Ca^{2+} sensitization mechanism. Treatment with Tempol decreased RVSP and was associated with normalization of vasoconstrictor responses, but not arterial remodeling.[82]

Histone acetylation is an epigenetic mechanism of oxidative stress. In a MCT rat model, multiple histone deacetylases (HDAC) isoforms were found to be upregulated in isolated pulmonary arteries. HDAC inhibitors attenuated NOX expression and reduced indices of PH.[83]

In FHR, treatment with oral dichloroacetate, an inhibitor of the mitochondrial pyruvate dehydrogenase kinase, reduced established PH and improved survival.[84] In PASMC culture, dichloroacetate increased SOD activity, eliminated nuclear HIF-1a translocation, and restored voltage gated potassium channel mRNA and protein expression.

Tetrahydrobiopterin (BH_4) is an essential cofactor that stabilizes the dimerization of eNOS and balances NO• and $O_2^{\bullet-}$ production. In the chronic hypoxia-induced PH rat

model, BH_4 administration reduced RVSP and pulmonary vascular muscularization with reduced $O_2^{\bullet-}$ production and an increase in cGMP.[85] In another study, administration of L-arginine and/or BH_4 in pneumonectomy and MCT-induced rats lowered PAP and increased pulmonary vascular elasticity. BH_4 lowered asymmetric dimethylarginine levels and downregulated eNOS mRNA expression.[86] In hypoxic piglets, L-citrulline, a precursor for L-arginine, increased NO• production by recoupling eNOS and decreased PVR.[87]

An increase in NOX-1 and a decrease in SOD1 was found in hypoxic newborn piglets. Administration of SOD mimetic + polyethylene glycol-catalase diminished responses to acetylcholine in pulmonary arteries in hypoxic piglets.[88] In addition, Apocynin, which is an ortho-methoxy-substituted catechol derived from the plant Picrorhiza kurroa, selective for inhibition of NOX-2, was shown to ameliorate PH in these hypoxic pigs. In another study, intratracheal delivery of apocynin to lambs with persistent PH of the newborn improved oxygenation and reduced PA contractility.[89] Apocynin also increased PA eNOS expression, NO• levels and BH_4 to BH_2 ratio.

Oltipraz, a potent Nrf2 activator, significantly attenuated RVH and pulmonary vascular remodeling in hypoxic mice.[52]

HUMAN STUDIES

Studies of ROS in humans with PAH are limited. In ex vivo studies utilizing human PAECs, there is evidence of DNA and protein oxidation in biopsies of the pathognomonic plexiform lesions.[90] Nitrotyrosine and 8-hydroxyguanine, markers of oxidative stress, were found to be elevated in lung tissue of patients with severe PH.[90] In 8 PAH patients undergoing transplant, small pulmonary arteries in the explanted lungs had reduced expression of mitochondrial electron transport chain complex I and were deficient in SOD2 expression/activity.[47] Treatment of the ex-vivo PASMCs with dichloroacetate improved

mitochondrial function and increased SOD activity.[47] In addition, treatment of hypoxic human PASMCs with siRNA directed against NOX-4 decreased PASMC proliferation and generation of ROS.[38]

A pilot study of sera from patients with systemic sclerosis (SSc) with and without PAH, and normal controls was applied to human PASMCs. ROS were assessed using dichlorodihydrofluoescein-diacetate, which showed significantly increased ROS levels in PASMCs treated with sera from SSc-PAH patients compared to SSc and healthy controls. In that same study, PASMCs were treated with the NOX specific inhibitor (NOX2ds-tat) before treatment with SSc-PAH sera which effectively reduced induction of ROS.[91]

In 12 iPAH patients, plasma levels of L-arginine, as measured by liquid chromatography correlated to right atrial pressure (RAP), cardiac output (CO), cardiac index (CI), mixed venous oxygen saturations (MVO_2 sats), 6MW distance and WHO functional class.[92]

High altitude PH was evaluated in 26 subjects showing a progressive decrease in arterial nitrite and S-nitrosothiols by ozone-based chemiluminescence and ascorbate free radical by EPR spectroscopy which correlated to the pulmonary artery systolic pressure (PASP) on ECHO.[93] Increased PASP at high altitude was associated with a reduction in pulmonary NO• bioavailability.

In a group of 347 patients, the ratios of cysteine and its oxidized form, cystine, and glutathione and its oxidized glutathione disulfide were measured in plasma by high performance liquid chromatography. For each 1% increase in plasma cystine, PASP on ECHO increased by 16%.[94] Thus, plasma aminothiols, which represent non-free radical oxidative stress burden, were associated with increases in PASP.

Measurements of peripheral vasoreactivity in 18 patients with severe iPAH showed significant impairment compared to 36 age- and sex-matched controls.[95] In addition to confirming these findings of abnormal flow-mediated dilation in patients with PH, oxidative

stress was assessed in 14 iPAH patients and 14 controls by measuring MDA levels, a marker for lipid peroxidation, XO and endothelial-bound SOD activity. Flow mediated dilation was assessed by ultrasound of the brachial artery, which was abnormal in the patients with PH. PAH patients showed increased MDA levels and XO activity and decreased endothelial SOD activity. Treatment with prostanoid correlated with endothelial SOD activity.[96] These measurements were taken from antecubital venous blood, suggesting that oxidative stress and endothelial dysfunction in PAH is not confined to the pulmonary vessels but involves the systemic vasculature as well.

Urinary F_2-isoprostanes are one of the most stable markers of lipid peroxidation. In 110 consecutive patients with incident PAH (treatment-naïve), venous blood samples and urine samples were obtained within 24 hours before RHC. Urinary isoprostane was determined by gas chromatography-mass spectrometry. Patients were followed for 3 years. In multivariate analysis, urinary 15-F_2-isoprostane was the only biomarker that was independently associated with increased adjusted hazard of death.[97] In another study, F_2-isoprostanes decreased in PAH patients after the initiation of treatment with the prostacyclin epoprostenol.[98]

In a small study, 12 patients with PAH confirmed by RHC and 12 normal controls underwent assessment of oxidative stress by measuring levels of MDA, which was significantly elevated in patients with PAH.[99]

In 45 patients with Group 1 PAH or CTEPH, peripheral venous samples were collected.[100] Total antioxidant capacity was measured based on inhibition of the absorbance of the radical cation of 2,2'-azino-bis (3-ethylbenzothiazoline 6-sulphonate), which is formed by activation of metmyoglobin with H_2O_2. Lipid peroxidation was also assessed by measuring serum levels of thiobarbituric acid reactive substances, including MDA, measured by colorimetric absorbance. SOD activity was measured as a percent inhibition of the tetrazolium salt WST-1 to water-soluble WST-1 formazan.

Catalase activity was assayed with the OxiRed probe. After following patients for 13 months, MDA, total antioxidant capacity, and catalase activity were associated with clinical deterioration.

Coenzyme Q functions as an electron carrier in the inner mitochondrial membrane and has an important role as an antioxidant which inhibits the initiation and propagation of lipid and protein oxidation. In a small pilot study, Coenzyme Q supplementation in 8 patients with PAH did not change redox parameters such as lactate, pyruvate and reduced or oxidized glutathione or clinical variables such as 6MW or BNP levels.[101]

In a phase 2a study, PAH patients were treated with AT-877, fasudil hydrochloride, a Rho-kinase inhibitor (n=9) or placebo(n=11).[102] There was a trend toward improvement in mean PAP, PVR and CI in patients treated with fasudil.

Oxidative stress was assessed in a case control study of 35 patients with PH.[103] There was no difference in the derivative of reactive oxygen molecules but asymmetric dimethylarginine, an endogenous NOS inhibitor, was found to be associated with pulmonary hemodynamics in PAH patients. In this particular study, the investigators measured the derivative of ROS by using an automatic chemical analyzer that assesses oxidation of serum hydroperoxides to alkoxyl and peroxyl radicals that react with chromogen. Although this method provides insight into the overall oxidative environment, it does not directly measure specific ROS. In our project described herein, we utilized EPR Spectroscopy, the reference-standard for detecting free-radicals, to directly measure levels of $O_2^{\cdot-}$ in patients with PH.

HYPOTHESIS:

PAH is a devastating cardiovascular disease that results in significant limitations in functional capacity, reduced quality of life, and markedly decreased life expectancy. Increased levels of ROS associated with the development of oxidative stress, along with induction of proliferative pathways, altered metabolic signaling, genetic mutations, and increased inflammation are believed to contribute to the pathogenesis of vascular remodeling in PAH. Despite advances in medical therapy including those that target prostacyclin, ET, and NO• pathways, prognosis remains poor. Notably, although NO• and ROS are closely linked and are often associated with numerous cardiovascular diseases, including PAH, none of the currently available PAH therapies directly target specific ROS.

There is consistent evidence for increased levels of ROS in experimental animal models of PH and some evidence in human PASMCs and PAECs[90,104] from patients with PAH. Oxidative stress in Group 1 PAH and CTEPH patients has been assessed using nonspecific methods such as measuring oxidatively modified nucleotides like 8-hydroxyguanine, lipid peroxidation products like MDA and F2-isoprostanes, and protein carbonyl formation.[105] Other studies have used dichlorodihydrofluorescein-diacetate, a non-specific fluorogenic probe sensitive to oxidation, or have measured cysteine/cystine or glutathione/glutathione disulfide ratios.[91,94,97,106,107] Although these previous studies reported increased levels or activation of these oxidative stress biomarkers, these markers do not provide insight into the specific ROS mediating the oxidative stress environment. There are no data assessing the contribution of $O_2^{\bullet-}$ specifically in the pathogenesis of PAH human patients. Our group and others have shown that $O_2^{\bullet-}$ is a key signaling intermediate in various models of hypertension.[108-110] In our project described herein, we utilized EPR spectroscopy, considered by many to be the gold-standard for detecting free radicals, to quantify levels of the free radical ROS, $O_2^{\bullet-}$.[70,71] **We**

hypothesize that superoxide-driven oxidative stress is pathogenic in PAH and that levels of $O_2^{\bullet-}$ would be elevated in whole blood from patients with PAH and would correlate with markers of disease severity as assessed by symptoms, functional capacity and hemodynamics. We hypothesize that patients with worse PAH hemodynamically, that is, with a higher PVR, would have higher $O_2^{\bullet-}$ levels and that $O_2^{\bullet-}$ levels would be inversely correlated to pulmonary artery capacitance (PAC) and to MVO_2 sats. BNP is a blood test that assesses the release of natriuretic peptide from the atria. It is often elevated in patients with atrial stretch, which occurs as the right heart is failing from PH. We hypothesize that $O_2^{\bullet-}$ levels would be elevated in patients with worse right heart failure as assessed by increased levels of BNP. 6MW distance is often used as a surrogate endpoint in studies of PAH. It is a commonly used test for the objective assessment of functional exercise capacity. We hypothesize that $O_2^{\bullet-}$ levels would be higher in patients with worse functional capacity as measured by 6MW, that is, inversely related to the distance. We also hypothesize that patients with increased oxidative stress as assessed by increased levels of $O_2^{\bullet-}$ would have increased REVEAL risk scores.

Our study is innovative as it directly measures $O_2^{\bullet-}$ in human patients with PAH as opposed to nonspecific biomarkers of oxidative stress, as previously performed. We examine $O_2^{\bullet-}$ as a novel and specific biomarker of PAH to elucidate potential mechanisms of specific ROS in the pathogenesis of PAH.

CHAPTER 2 METHODS:

1. PILOT STUDY

Initially, we did a pilot study to determine whether $O_2^{\cdot-}$ levels from pulmonary arterial blood correlate with peripheral venous blood. We evaluated a prospective, observational cohort of 22 patients with suspected PAH undergoing diagnostic RHC.

MATERIAL AND METHODS

Patient population:

Twenty-two patients greater or equal to 19 years of age, were referred to the subspecialty PH clinic at the University of Nebraska Medical Center in Omaha, Nebraska. Based on history, physical examination, laboratory data, pulmonary function tests, imaging studies (chest radiograph, computerized tomography of the chest, ventilation perfusion scans), and echocardiographic measures, the primary investigator determined patients with high probability of PAH. The patients were then sent for RHC as a confirmation of the diagnosis and classification of PH. The 22 patients gave written consent to participate in the study after the Principal Investigator obtained informed consent in accordance with the Declaration of Helsinki.

Exclusion criteria included patients with strong clinical suspicion of group 2, 3, and 5 PH and patients with an established diagnosis of PAH. The local Institutional Review Board (IRB) approved the study (protocol # 598-17-EP).

Healthy subjects from a previous investigation into $O_2^{\cdot-}$ levels in healthcare workers exposed to ionizing radiation versus normal subjects were used for comparison in this pilot.[73] The healthy subjects were not taking any medications, were nonsmokers, and did not have a chronic medical problem based on patient-completed questionnaires.

Study protocol:

This was a prospective pilot study. From January to September of 2018, patients with suspicion of PAH were sent for RHC. Peripheral blood was collected immediately prior to the RHC. PA blood was collected during the RHC. Blood was processed immediately for the measurement of $O_2^{\cdot-}$ levels using EPR Spectroscopy. After RHC, patients were classified as having PAH (mPAP > 25 mmHg, PAOP < 15 mmHg and PVR > 3 WU), pulmonary venous hypertension (mPAP > 25 mmHg, PAOP > 15mmHg and PVR < 3 WU), or no PH (mPAP < 25 mmHg).[8]

Pulmonary artery blood procurement:

A 7 French thermodilution balloon-tipped catheter was inserted percutaneously, under local anesthesia, into the right internal jugular vein under ultrasound guidance and advanced to the right atrium (RA), right ventricle (RV), PA, and to the PAOP position. Pressure transducers were balanced against atmospheric pressure, and the zero reference levels for recordings were 5 cm below the sternal angle. Mean pressures were obtained by electronic damping. Confirmation of location of catheter into the PA was done by direct visualization under fluoroscopic guidance and pressure waveform analysis. Once in the PA, 2 mL of blood was obtained in an EDTA tube and transported immediately to the lab for processing.

Peripheral venous blood procurement:

Minutes before the patient was transferred to the catheterization laboratory, the registered nurse in charge of the patient in the pre-procedural area, collected 2 mL of blood from an antecubital vein from either arm into an EDTA tube. The blood samples were transported immediately to the lab for processing and analysis of the sample.

Superoxide measurement:

Superoxide was measured by EPR Spectroscopy using a Bruker e-scan spectrometer, as previously described.[73] 200 µl of blood was immediately incubated for 60 minutes at 37°C with 200 µM of the $O_2^{\bullet-}$-sensitive EPR spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Noxygen Science Transfer & Diagnostics, Germany). The CMH spin probe was prepared in buffer (Krebs-Hepes buffer (KHB) containing 25 µM deferoxamine methane-sulfonate salt (DF) and 5 µM sodium diethyldithio-carbamate trihydrate (DETC) at pH 7.4. Immediately after incubating blood with CMH, 100 µL of the treated blood was placed into a 1 mL syringe and was flash frozen. The frozen sample was then transferred to a glass liquid nitrogen finger dewar, which was inserted into the cavity of a Bruker e-Scan EPR Spectrometer (Bruker, Germany) for data acquisition. The following EPR spectrometer settings were used: field sweep width, 100.0 G; microwave frequency, 9.75 kHz; microwave power, 1.10 mW; modulation amplitude, 5.94 G; conversion time, 10.24 ms; time constant, 40.96 ms. Superoxide levels measured by the spectrometer are expressed as EPR Spectrum Amplitude in arbitrary units (a.u.).

PAH disease severity was assessed using the following measures:

Functional assessment: Subjects underwent World Health Organization (WHO) functional class assessment.[8] Six-minute walk was performed according to American Thoracic Society protocol.[111]

Serology: Measurement of B-type natriuretic peptide (BNP) was obtained in PAH patients as clinically indicated.

Hemodynamic assessment: The following echocardiographic measurements were assessed: RA size measured by planimetry of the RA cavity in the apical four-chamber view at end-systole and indexed to the patient's body surface area. RV size

and function assessed qualitatively in the apical four-chamber view at end-diastole and by Tricuspid Annular Plane Systolic Excursion (TAPSE), and estimated PASP as assessed by the regurgitant jet across the tricuspid valve. RHC was performed according to a standard clinical protocol with assessment of RAP, PAPs, PAOP, and cardiac output (CO) by Fick. Calculations of cardiac index (CI), PVR, and PAC were performed. PAC is a measure of the distensibility of the pulmonary artery. It is calculated as: Stroke volume/Pulmonary Pulse Pressure. Previous studies have shown that PAC may correlate with mortality.[112]

SAMPLE SIZE

Sample size was determined by setting a desired, minimum detectable correlation between continuous measures of 0.60. A sample size of 20 achieves 80% power to distinguish a correlation of 0.58 from no correlation using a two-sided hypothesis test at a significance level of 0.05.

2. CASE CONTROL STUDY

After our pilot study showed a correlation between $O_2^{\cdot-}$ levels in PA and peripheral blood, we then proceeded to perform a case-control study to measure $O_2^{\cdot-}$ levels in peripheral venous blood in patients with PAH and 18 sex-matched controls to address our hypothesis that circulating $O_2^{\cdot-}$ levels would be significantly higher in PAH patients vs. controls and would be associated with physiologic and hemodynamic measures of disease severity.

MATERIAL AND METHODS

Human Subjects:

Patients were screened in our subspecialty PH clinic

Inclusion Criteria:

Patients with incident (treatment-naïve) or prevalent PAH (Group 1 or 4) by RHC:
mean PAP > 25 mm Hg
PAOP ≤ 15 mm Hg,
PVR > 3 Wood units

CTEPH was excluded or confirmed by ventilation-perfusion scan

≥ Nineteen years of age

Able to complete a 6MW

Able to speak and read English

Stable pharmacologic therapy per guidelines for past 30 days

Normal sex-matched controls who had no history of heart or lung disease, diabetes, or rheumatoid arthritis were recruited. Most volunteers worked on the UNMC campus.

All subjects were nonsmokers as cigarette smoke may affect antioxidant enzyme activities.[113]

Exclusion Criteria:

Pulmonary hypertension group 2, 3 or 5

Absence of RHC data or RHC data not consistent with PAH

Inability to perform 6MW

Case Control Study Protocol:

Informed consent was obtained in PAH clinic for PAH patients and in our research space for the controls in accordance with the Declaration of Helsinki. UNMC IRB# 786-16.

Review of medical history: A complete history was obtained, and physical exam was performed on PAH patients. A history was obtained, and a limited physical exam was performed on the normal subjects

Peripheral Venous Blood Procurement:

Subjects underwent a peripheral venous blood of 2cc placed in an ethylenediaminetetraacetic acid (EDTA) tube to measure $O_2^{\cdot-}$ levels. Blood was drawn prior to 6MW.

Superoxide measurement:

Superoxide was measured by EPR Spectroscopy using a Bruker e-scan spectrometer housed in the UNMC's EPR Spectroscopy Core, as previously described above.

PAH disease severity was assessed using the following measures:

Symptom assessment: Patients completed two questionnaires, including the Short Form-12 (SF-12)v1.0 and emPHasis 10.

a. SF 12: validated generic health-related quality of life questionnaire with 12 items to measure functional health and well-being from the patient's point of view. There are two summary scores: a mental component and a physical component.[114]
b. emPHasis 10: validated questionnaire of 10 items to assess health-related quality of life specifically in the context of PAH[115]

Functional assessment: Subjects underwent WHO functional class assessment.

6MW distance is often used as a surrogate endpoint in studies of PAH. It is a commonly used test for the objective assessment of functional exercise capacity. 6MW was performed according to American Thoracic Society guidelines.[111]

Serology: Measurement of BNP was obtained in PAH patients.

Hemodynamic assessment: Recent clinically indicated echocardiography and RHC of PAH patients were recorded. All control subjects underwent echocardiography. The following echocardiographic measurements were assessed: RA size measured by

planimetry of the RA cavity in the apical four-chamber view at end-systole and indexed to the patient's body surface area. RV size and function assessed qualitatively in the apical four-chamber view at end-diastole and by TAPSE, and estimated PASP as assessed by the regurgitant jet across the tricuspid valve. RHC was performed according to standard protocol as described above with assessment of RAP, PAPs, PAOP, and CO by Fick. Blood samples were taken from the PA for mixed venous oxygen saturations (MVO_2 sats). MVO_2 sats have been shown to predict mortality in PAH.[116] Calculations of CI, PVR, and PAC were performed.

Risk assessment: The subject's REVEAL risk score was calculated.

Table 1: Schedule of events

	SF 12	EmPHasis 10	6MW	WHO FC	H & P	ECHO	RHC	REVEAL score	$O_2^{\bullet-}$
PAH	X	X	X	X	X	Chart review	Chart review	X	X
Controls	X	X	X	X	X	X			X

SF-12 Short Form 12 and EmPHasis 10 quality of life questionnaires
H&P History and Physical; WHO FC World Health Organization Functional Class
ECHO echocardiography; RHC right heart catheterization

STASTICAL ANALYSIS

For the pilot study, sample size was determined by setting a desired, minimum detectable correlation between continuous measures of 0.60. A sample size of 20 achieves 80% power to distinguish a correlation of 0.58 from no correlation using a two-sided hypothesis test at a significant level of 0.05. Samples (n=22) were obtained with the understanding that some of the samples would come from patients that do not have PAH demonstrated during the RHC. The assumption of normality was visibly checked

using histograms and QQ-plots. Analyses and plots were conducted using R version 3.5.0 statistical computing software

For the case control study, summary statistics were computed for the primary outcomes and demographic variables presented as means ± standard deviation. The EPR amplitude, 6MW distance and hemodynamics, for the affected and control groups were compared using a Welch's two-sample t-test. If the assumptions of the t-test are violated, comparisons were made using Wilcoxon's sum rank test.

Sample Size and Power: Group sample sizes of 18 controls and 36 cases achieve 80.1% power to detect a difference of 0.7×10^6 a.u. based on prior studies[73] assuming standard deviations of 0.6×10^6 a.u. and 1.2×10^6 a.u. for control and affected groups, respectively, using an independent samples t-test and a significance level of 0.05.

Potential confounding variables PH group, drug pathway, BMI, age, and sex were explored using simple correlations (Pearson and Spearman), linear models, and Kruskal-Wallis tests, as appropriate.

CHAPTER 3 RESULTS:

1. PILOT STUDY:

Twenty-two patients referred to our subspecialty PH clinic underwent RHC for classification and confirmation of the diagnosis of PH. Baseline characteristics and RHC values of the subjects are summarized in Table 2. One subject was excluded due to extremely high $O_2^{\bullet-}$ levels attributed to coexistent chronic myelogenous leukemia.

Table 2: Baseline Characteristics of Pilot Study Cohort

	Pilot Study Cohort
Mean Age in Years	64.4 ± 14
Sex	5 Females (24%)
	16 Males (76%)
Race	White 20
	Black 1
Body Mass Index (kg/m2)	30.8 ± 10.6
DLCO (Percent Predicted)	49 ± 20.5
6MW Distance (meters)	301.0 ± 80.0
ECHO findings:	
RA Enlargement n (%)	9 (42.8%)
PASP (mm Hg)	62.2 ± 17.6
RV dysfunction n (%)	12 (57.0%):
	8 mild
	1 moderate
	3 severe
TAPSE (cm)	2.1 ± 0.5
RV dilation	12 (57.0%)
RV hypertrophy	0
Pericardial effusion	2 (9.5%)
WHO functional class (n)	2: 5
	3: 14
	4: 2
REVEAL risk score	7.4 ± 2.6
BNP (pg/mL)	303.1 ± 526.0
Supplemental oxygen n (%)	11 (52.0%)
RHC data:	Mean ± SD
RAP (mm Hg)	10.0 ± 6.1
mean PAP (mm Hg)	41.0 ± 11.0
CO (L/min)	5.3 ± 1.5
CI (L/min/m^2)	2.7 ± 0.7
PAOP (mm Hg)	13.4 ± 6.8
PVR (Wood units)	5.8 ± 3.6
PAC (mL/mm Hg)	1.9 ± 1.0

Subjects were predominantly Caucasian, and the majority were male. Their mean age was 64 and they had mild obesity. They were ill as reflected by their severely reduced DLCO, elevated BNP, and poor functional class. Half of the subjects required supplemental oxygen.

Superoxide levels in whole blood obtained from the pulmonary artery correlate with superoxide levels obtained from peripheral venous blood. We assessed whether levels of $O_2^{\cdot-}$ obtained from whole blood from the pulmonary artery at the time of RHC correlated with $O_2^{\cdot-}$ levels obtained from a peripheral vein just prior to the procedure. As shown in **Table 3** and **Figure 1**, there was a significant correlation.

Table 3: Correlation of superoxide levels in the pulmonary artery versus peripheral vein

Correlation and R-Square

Correlation	R-Square	F-Value	Pr>F
0.77	0.59	24.21	<0.0001

Based on this sample of 21 subjects, the correlation between peripheral vein and pulmonary artery $O_2^{\cdot-}$ levels was 0.77 ($p < 0.0001$; $R^2 = 0.59$). The assumption of normality was visibly checked using histograms and QQ-plots. Analyses and plots were conducted using R version 3.5.0 statistical computing software.

Figure 1: Correlation of $O_2^{\cdot-}$ levels in pulmonary artery with peripheral vein

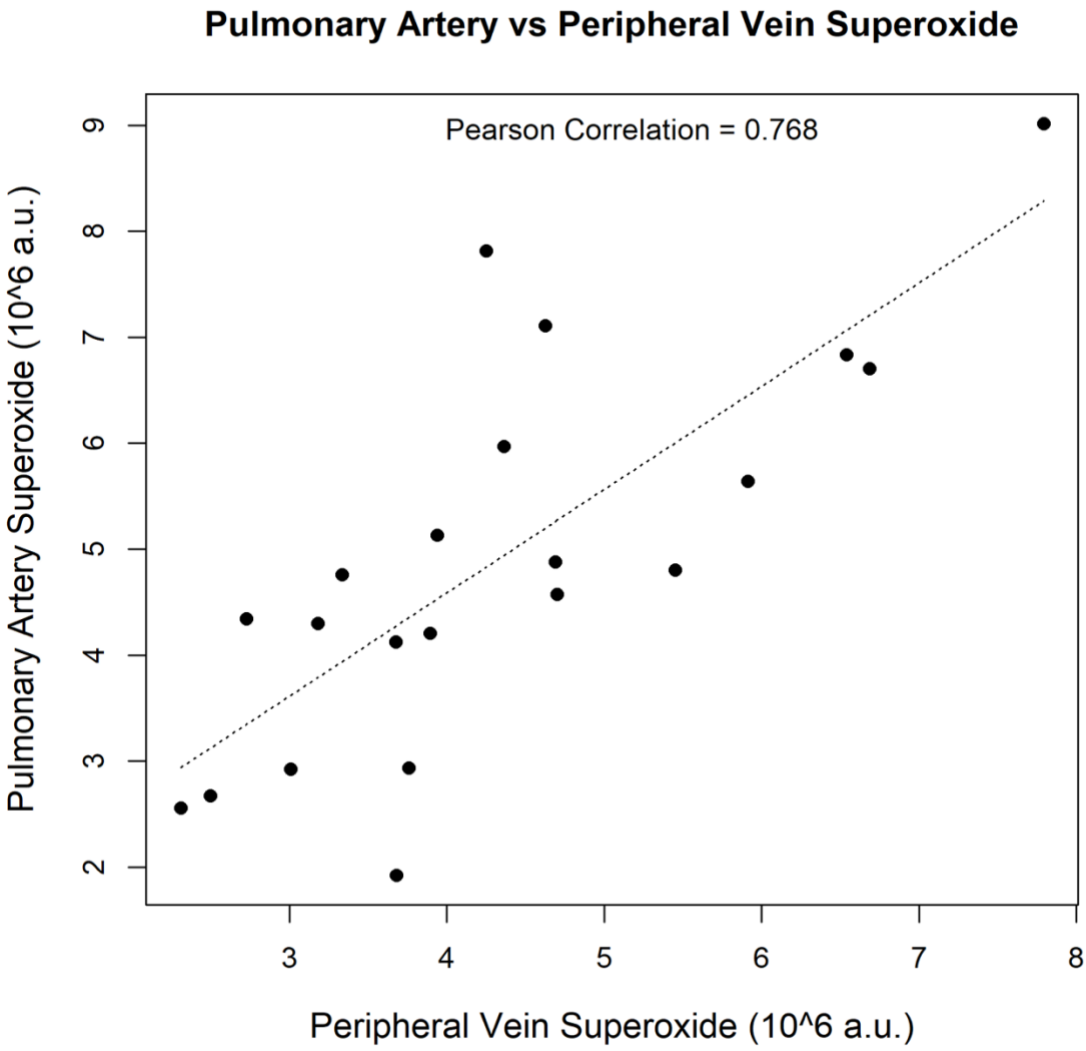


Figure 1: Scatter plot showing relationship between $O_2^{\cdot-}$ levels in whole blood obtained from the pulmonary artery and peripheral vein. The individual dots (n=21) represent the plotted values obtained from each of the individual subjects. The line represents the best fit for the correlation.

Additionally, a Bland-Altman agreement plot was constructed (**Figure 2**). The mean difference was 0.58 (1.18) with lower and upper limits of agreement of -1.89 and 3.05, respectively.

Figure 2: Agreement plot of pulmonary and peripheral $O_2^{\cdot-}$ levels

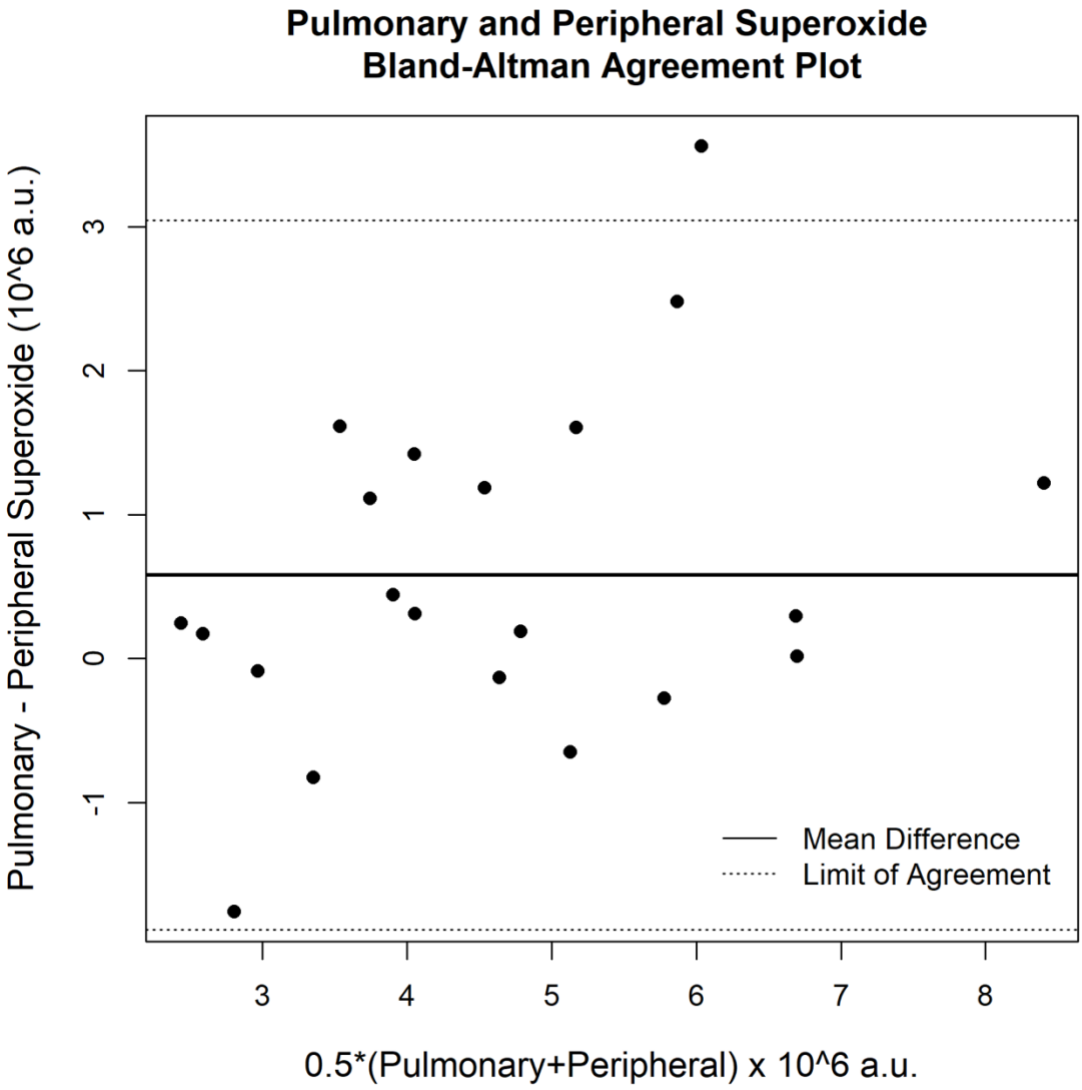


Figure 2: Pulmonary artery and peripheral vein Bland-Altman agreement plot. This plot shows the agreement between the two quantitative measurements of $O_2^{\cdot-}$ levels from whole blood collected from the pulmonary artery and the peripheral vein. Individual dots (n=21) are the difference of the two measurements for each subject plotted against their mean. The dotted lines are the limits of agreement (+/- 2 standard deviations) and 95% of the data points lie within 2 standard deviations of the mean difference.

Superoxide levels are elevated in patients subjected to RHC compared to healthy individuals: To examine potential differences in $O_2^{\cdot-}$ levels in subjects undergoing RHC

for evaluation of PH versus healthy subjects, we compared the EPR spectrum amplitude obtained from whole blood collected from healthy individuals, as we previously reported,[73] to whole blood from our patients who received RHC for the diagnosis of PH. As shown in the representative EPR spectra (Figure 3A) and summary data (Figure 3B), $O_2^{\bullet-}$ levels, reported as the EPR spectrum amplitude, in whole blood from our subjects undergoing RHC were elevated compared to $O_2^{\bullet-}$ levels in healthy subjects.

Figure 3: Levels of $O_2^{\bullet-}$ in Patients undergoing RHC versus historical normal

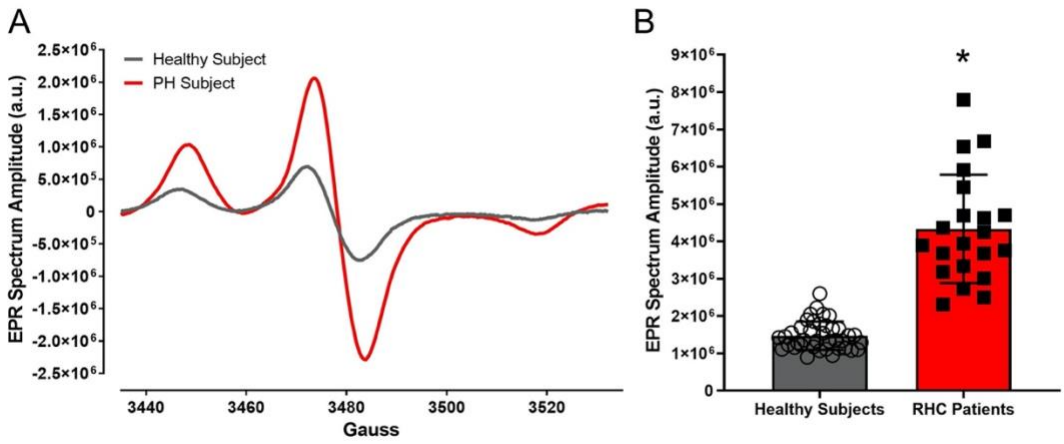


Figure 3: Superoxide ($O_2^{\bullet-}$) levels, as measured by EPR spectroscopy, are increased in patients that underwent RHC for diagnosis of PH compared to healthy subjects. A) Representative EPR spectra, indicative of $O_2^{\bullet-}$ levels, obtained from whole blood collected from a healthy subject and a PH patient B) Summary data showing elevated EPR spectra amplitude (i.e., $O_2^{\bullet-}$ levels) in patients that received RHC (n=21) versus healthy subjects (n=39). A.u. = arbitrary units *P<0.05

PH Group and Superoxide levels: Based on the RHC data shown in **Table 4**, 13 patients were found to have PAH (Groups 1 and 4), 4 patients had pulmonary venous hypertension (Group 2), 4 patients did not have PH.

Table 4: Right Heart Catheterization Data by PH Group

PH Group	PASP (mmHg)	PA Diastolic Pressure (mmHg)	Mean PAP (mmHg)	Cardiac Output (L/min)	PAOP (mmHg)	PVR (WU)
1 (n=9)	68.0 ± 15.0	26.0 ± 8.9	42.5 ± 9.2	4.9 ± 1.3	10.8 ± 3.6	6.9 ± 3.0
2 (n=4)	76.5 ±12.4	27.8 ± 3.8	49.0 ± 10.5	5.5 ± 1.4	21.8 ± 2.5	5.0 ± 1.5
4 (n=4)	64.0 ± 20.1	20.8 ± 10.5	39.0 ± 12.0	5.3 ± 1.5	7.0 ± 2.8	7.0 ± 5.5
no PH (n=4)	40.2 ± 6.1	17.0 ± 5.0	28.0 ± 7.0	6.1 ± 2.2	17.2 ± 8.1	1.9 ± 0.9

To better understand potential differences in $O_2^{\bullet-}$ levels between the different groups of PH, we separated the patients that underwent RHC by their PH diagnosis and compared $O_2^{\bullet-}$ levels across the groups (**Table 5** and **Figure 4**). The mean $O_2^{\bullet-}$ levels in peripheral venous blood from patients with PH (i.e., Group 1, 2, and 4) was 4.5 x 10^6 a.u. with a standard deviation of 1.6 x 10^6 and standard error of 0.4 x 10^6, which was elevated when compared to healthy subjects (n=39; $O_2^{\bullet-}$ level of 1.5 x 10^6 a.u.), as we previously reported.[73] When we stratified the PH patients further, the mean $O_2^{\bullet-}$ level in the 13 patients with PAH (Groups 1 and 4) measured from the pulmonary artery was 5.2 x 10^6 a.u. and the peripheral vein was 4.7 x 10^6 a.u. The mean $O_2^{\bullet-}$ level in the 4 patients with pulmonary venous hypertension (Group 2) was 4.9 x 10^6. In patients with no PH, who underwent RHC (n=4), the mean $O_2^{\bullet-}$ level measured from the PA was 4.0 x 10^6 a.u which is lower than the $O_2^{\bullet-}$ levels in patients with PAH (Groups 1 and 4). Although numbers in each subgroup of subjects undergoing RHC were small, there was a trend of elevated $O_2^{\bullet-}$ levels in subjects with Group 1 PAH, and Group 4 CTEPH. It should be noted that $O_2^{\bullet-}$ levels in patients with no PH were higher than those we previously measured in healthy subjects. However, despite not having PH by RHC, these patients were not healthy; they had significant dyspnea and exercise limitation, thus their referral for RHC. With a limited number of subjects in each group of patients that underwent RHC, no definitive conclusions can be made.

Table 5: EPR Spectrum Amplitude (in arbitrary units a.u.) by PH Group			
PH Group	n	Mean $O_2^{\bullet-}$ (x10^6a.u.) (Stdev)	Median (x 10^6a.u.) (IQR)
Group 1	9	5.0 (2.0)	4.8 (4.1 – 5.6)
Group 2	4	4.9 (2.1)	4.5 (3.9 – 5.5)
Group 4	4	5.6 (1.5)	5.6 (4.3 – 6.9)
No PH	4	4.0 (1.9)	4.0 (2.7 – 5.3)

Stdev standard deviation; IQR Interquartile range

Figure 4: Levels of Superoxide by PH group as diagnosed at RHC

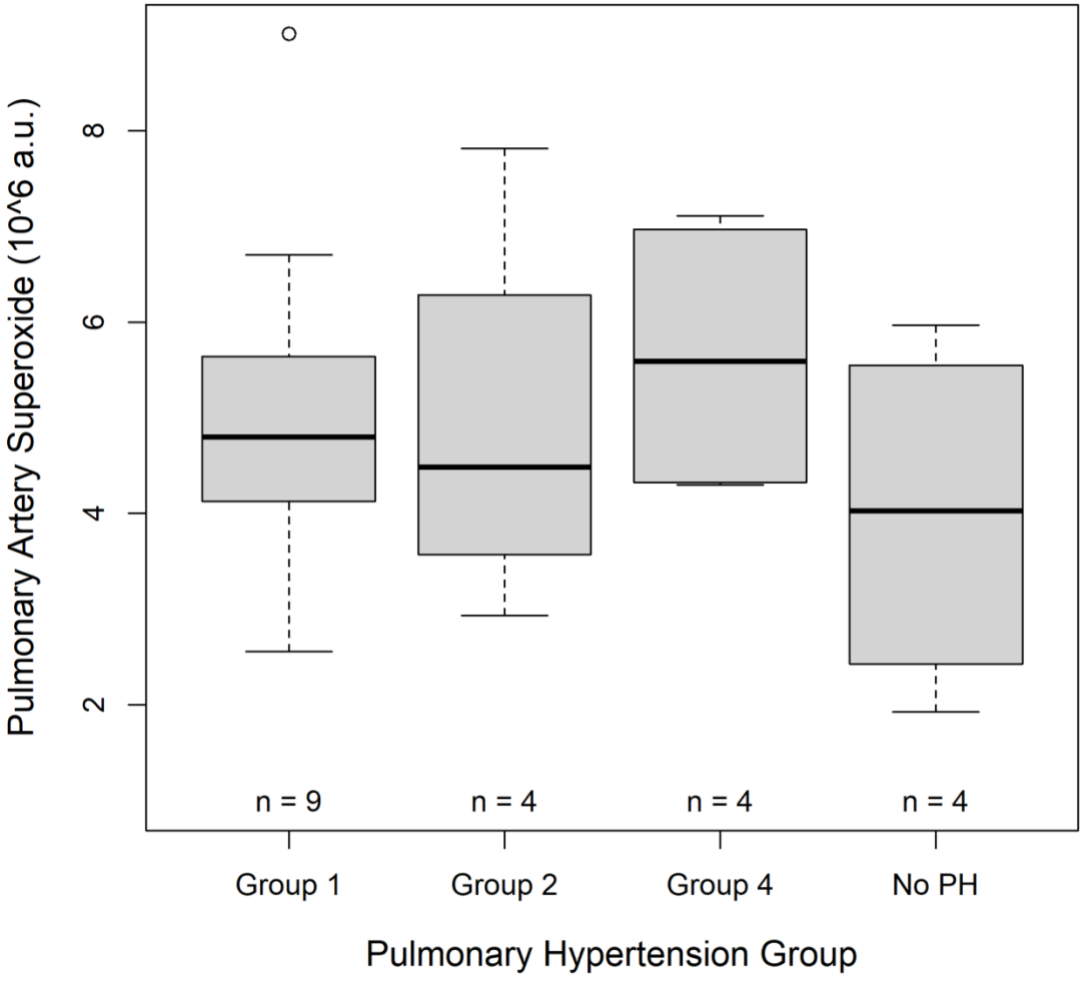


Figure 4: Box plot depicting superoxide levels by PH group. This graph shows the median (bold line) and the 25-75th percentile of $O_2^{\bullet-}$ levels in whole blood collected from the pulmonary artery in each of the groups of PH identified on RHC.

2. CASE CONTROL STUDY:

We then collected peripheral venous blood in 18 control subjects and 71 patients with PAH. Baseline characteristics of the two groups are reported in **Table 6.**

Table 6: Baseline Characteristics of Case Control Study Cohort

	PAH Subjects	Controls
Age in Years	61.8 ± 16.1	35.2 ± 8.5
Sex n (%)	Females 48 (67.6%) Males 23 (32.4%)	Females 12 (66.7%) Males 6 (33.3%)
Race n (%)	White 63 (89.0%) Asian 1 (1.4%) Black 7 (9.8%)	White 10 (55.5%) Asian 7 (38.9%) Black 1 (5.5%)
Body Mass Index (kg/m^2)	32.5 ± 8.9	28.6 ± 7.4
DLCO (Percent Predicted)	52.1 ± 22.2	ND
6MW Distance (meters)	300.7 ± 126.7	522.5 ± 64.9
ECHO findings:		
Right Atrial Enlargement n (%)	42 (59.1%)	0
PASP (mm Hg)	57.6 ± 23.7	24.4 ± 1.8
RV dysfunction n (%)	44 (62.0%): 12 mild 10 moderate 7 severe	0
TAPSE (cm)	2.1 ± 0.5	2.2 ± 0.4
RV dilation or hypertrophy	44 (62.0%) or 6 (8.4%)	0
Pericardial effusion present	8 (11.3%)	0
WHO Functional Class	1: 5 2: 35 3: 31 4: 0	1: 18
SF-12 PCS-12 **MCS-12**	34.0 ± 11.6 49.7 ±13.3	56.4 ± 1.9 53.5 ± 8.4
EmPHasis 10	23.8 ± 11.3	0.7 ± 1.5

Data presented as mean ± standard deviation. ND Not Done

There were differences in age between the two groups with the PAH patients being older. There were more Asians in the healthy control group and more blacks in the PAH cohort. PAH patients were heavier with a mean BMI in the obese range whereas the mean BMI

of the controls was in the overweight category. The other differences were expected based on the diagnosis of PAH such as lower 6MW distances, echocardiographic findings and emPHasis 10 scores. There was a difference in the SF-12 between the two groups with a much lower score in the physical component and a slight decrease in the mental component in the patients with PAH. Characteristics of the PAH patients are further delineated in **Table 7**.

Table 7: Characteristics of PAH patients

WHO Group	**Group 1: 58** Congenital Heart: 3 CTD: 12 Familial: 2 Portopulmonary: 1 Medication: 4 Idiopathic: 36 **Group 4 (CTEPH): 13**
REVEAL risk score	7.4 ± 2.6
Duration of disease (months)	1-216
BNP (pg/mL)	162.4 ± 259.4
Supplemental oxygen (n)	38 (53.5%)
PAH Medications (n)	Prostacyclin 29 Endothelin Receptor Antagonist 49 cGMP stimulator 15 Phosphodiesterase inhibitor 49 Calcium channel blocker 9
RHC data:	
RAP (mm Hg)	9.5 ± 4.5
mean PAP (mm Hg)	44.8 ± 13.1
CO (L/min)	4.9 ± 1.5
CI (L/min/m2)	2.5 ± 0.7
PAOP (mm Hg)	11.5 ± 5.0
PVR (Wood units)	7.9 ± 4.8
PAC (mL/mm Hg)	1.59 ± 0.8
MVO_2 saturations	61.4 ± 6.1

Data presented as mean ± standard deviation.

The majority of patients were categorized as Group 1 with predominantly idiopathic or CTD related etiologies. Most patients were prevalent (61) with only 10 incident patients. Approximately half of the patients required supplemental oxygen. The majority (72%) were on combination PAH therapy.

Superoxide in patients with PAH and controls: We found no significant difference in circulating venous $O_2^{\bullet-}$ levels between patients and controls (**Figure 5**). The mean EPR spectrum amplitude, which is directly proportional to the amount of free radicals in the sample (i.e., $O_2^{\bullet-}$), from PAH patients was $5.5 \times 10^6 \pm 1.6 \times 10^6$ a.u. The mean EPR spectrum amplitude in the control group was $4.9 \times 10^6 \pm 2.1 \times 10^6$ a.u. ($p = 0.23$).

Figure 5: Superoxide levels in pulmonary hypertension and control groups

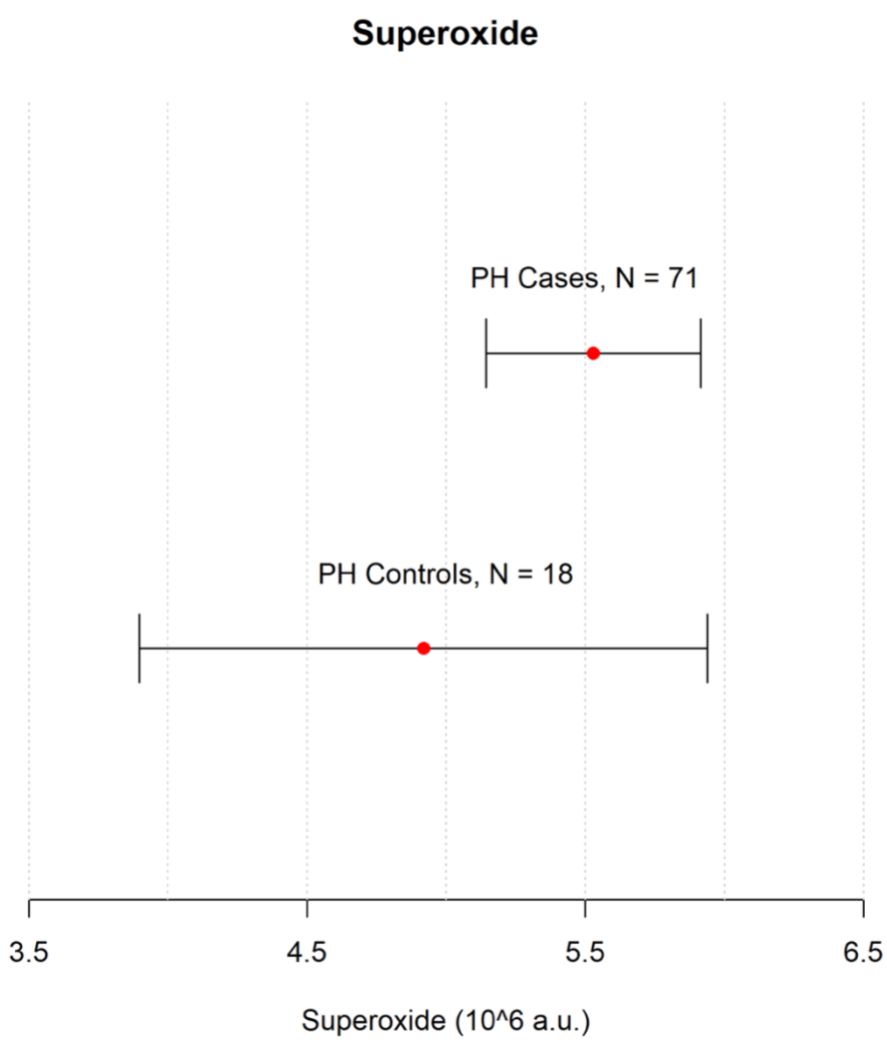


Figure 5: **Superoxide levels in whole blood collected from PAH patients and healthy (control) individuals.** This graph shows the mean ± standard deviation of $O_2^{\bullet-}$ levels measured by EPR spectroscopy in patients with PAH (PH cases) and healthy controls (PH Controls) with no significant difference between the two groups. A.u. = arbitrary units of EPR spectrum amplitude, which is directly proportional to the levels of free radicals in the sample. Effect size = 0.37; t statistic = -1.235, df = 21.944, p-value = 0.23.

Superoxide and PAH severity: In patients with PAH, we examined the relationship of $O_2^{\bullet-}$ levels with the hemodynamic variables measured at RHC including PVR and PAC, and MVO_2 sats, and clinical variables such as BNP and 6MW distance, and REVEAL risk score and found no significant correlation as shown in **Figure 6**.

Figure 6: Superoxide levels and clinical variables in PAH patients.

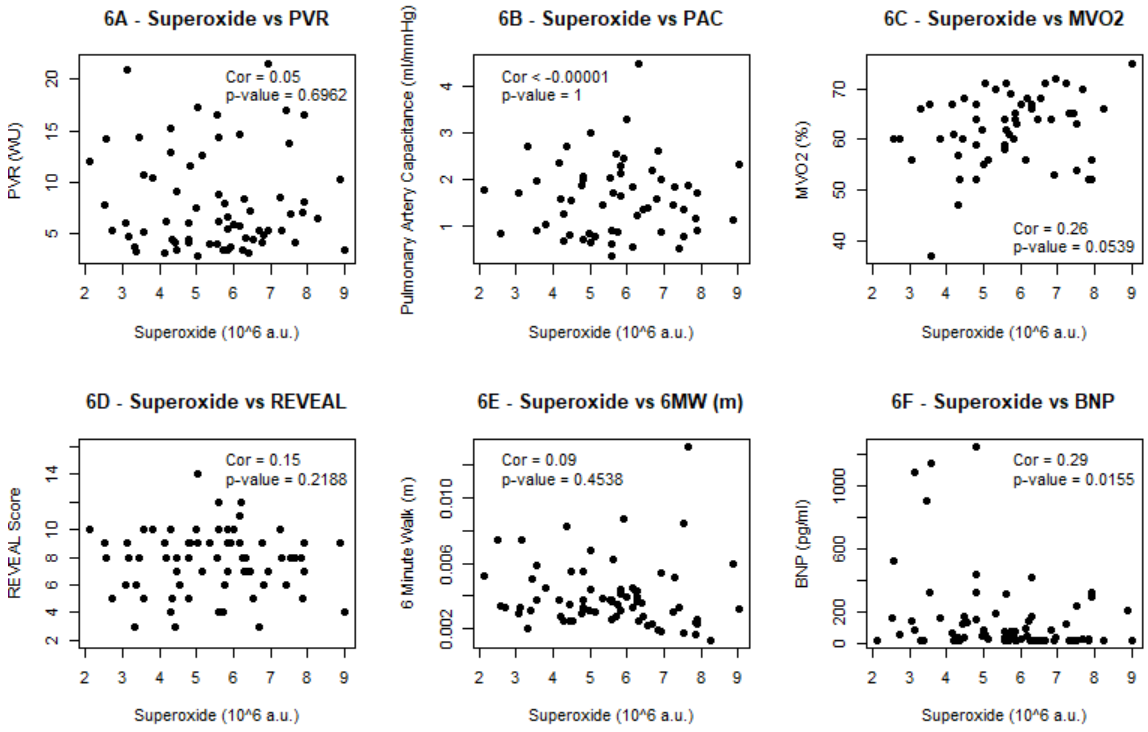


Figure 6: Scatterplots showing no correlation of superoxide with 6A: Pulmonary vascular resistance (PVR) 6B: Pulmonary arterial capacitance (PAC) 6C: Mixed venous oxygen saturations (MVO2) 6D: REVEAL risk score and 6E: 6-minute walk (6MW). 6F: This scatterplot shows there is a slight inverse correlation between $O_2^{\bullet-}$ and B-type natriuretic peptide (BNP).

As our results did not support our hypothesis, we evaluated multiple potential confounding factors. We first evaluated whether the differences in age could account for our null hypothesis as shown in **Table 8** and **Figure 7**. The sex variable was not significant (p-value = 0.40) and was dropped from the model. Superoxide was regressed vs age, cohort (PH or control) and age by cohort interaction. The interaction was significant,

indicating that the trend in $O_2^{\bullet-}$ vs time differs per cohort. In our PAH patients, $O_2^{\bullet-}$ levels decreased with increasing age whereas in this select group of normal controls, $O_2^{\bullet-}$ levels increased with age. However, our normals were young adults and we had no controls over the age of 60. Note, this analysis was repeated with an age matched subset; the interaction term was significant, and the sign and magnitude of the age coefficient were consistent with the model utilizing all data.

Table 8: Age and Superoxide Levels

PH Model	
With interaction	$\widehat{E(Superoxide)} = 7.25 - 0.03 * Age$
Control model	
With interaction	$\widehat{E(Superoxide)} = 1.71 + 0.09 * Age$

	Model with interaction				Model with no Interaction		
Parameter	Est.	St. Err.	p-value		Est.	St. Err.	p-value
Intercept	7.25	0.79	<0.0001		--	--	--
Control	-5.54	1.89	0.0043		--	--	--
Age	-0.03	0.01	0.0265		--	--	--
Age*Control	0.12	0.05	0.0174		--	--	--

Figure 7: Age and Superoxide levels

Figure 7: This scatterplot of $O_2^{\bullet-}$ versus age shows a difference in the trend in $O_2^{\bullet-}$ levels between PAH patients and controls. In PAH patients, $O_2^{\bullet-}$ levels decrease with age whereas in the controls, $O_2^{\bullet-}$increase with age.

We then evaluated whether the differences in BMI could be affecting our results as seen in **Table 9** and **Figure 8**. The sex variable was not significant (p-value = 0.9590) and was dropped from the model. Superoxide was regressed vs BMI, cohort (PH or control) and age by cohort interaction. The interaction was not significant, indicating that the trend in $O_2^{\bullet-}$ vs time does not differ per cohort.

Table 9: BMI and Superoxide levels

PH Model	
With interaction	$\widehat{E(Superoxide)} = 5.92 - 0.01 * BMI$
No interaction	$\widehat{E(Superoxide)} = 6.07 - 0.02 * BMI$
Control model	
With interaction	$\widehat{E(Superoxide)} = 6.34 - 0.05 * BMI$
No interaction	$\widehat{E(Superoxide)} = 5.39 - 0.02 * BMI$

	Model with interaction				Model with no Interaction		
Parameter	Est.	St. Err.	p-value		Est.	St. Err.	p-value
Intercept	5.92	0.71	<0.001		6.07	0.67	<0.001
Control	-0.01	0.02	0.565		-0.68	0.46	0.144
BMI	0.42	1.81	0.818		-0.02	0.02	0.400
BMI*Control	-0.04	0.06	0.531				

Figure 8: BMI and Superoxide levels

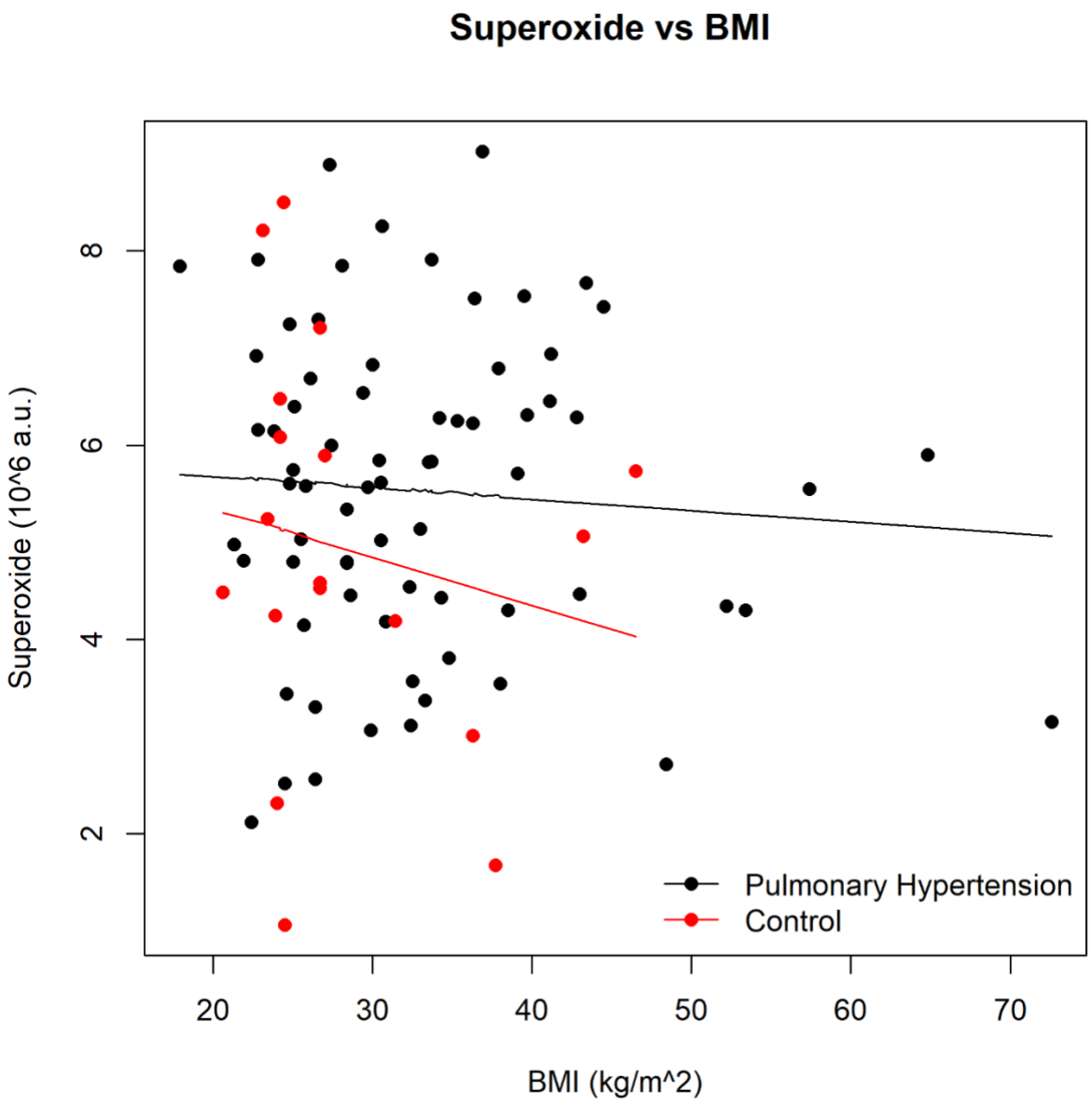


Figure 8: This scatterplot of $O_2^{\bullet-}$ versus BMI shows no significant association of BMI with $O_2^{\bullet-}$ levels in PAH patients or controls. The difference between the groups is not statistically significant.

PAH results from a multitude of pathologies so we evaluated whether the type of PAH may have affected our results as shown in **Figure 9** and **Table 10**.

Figure 9: Superoxide levels by PH etiology

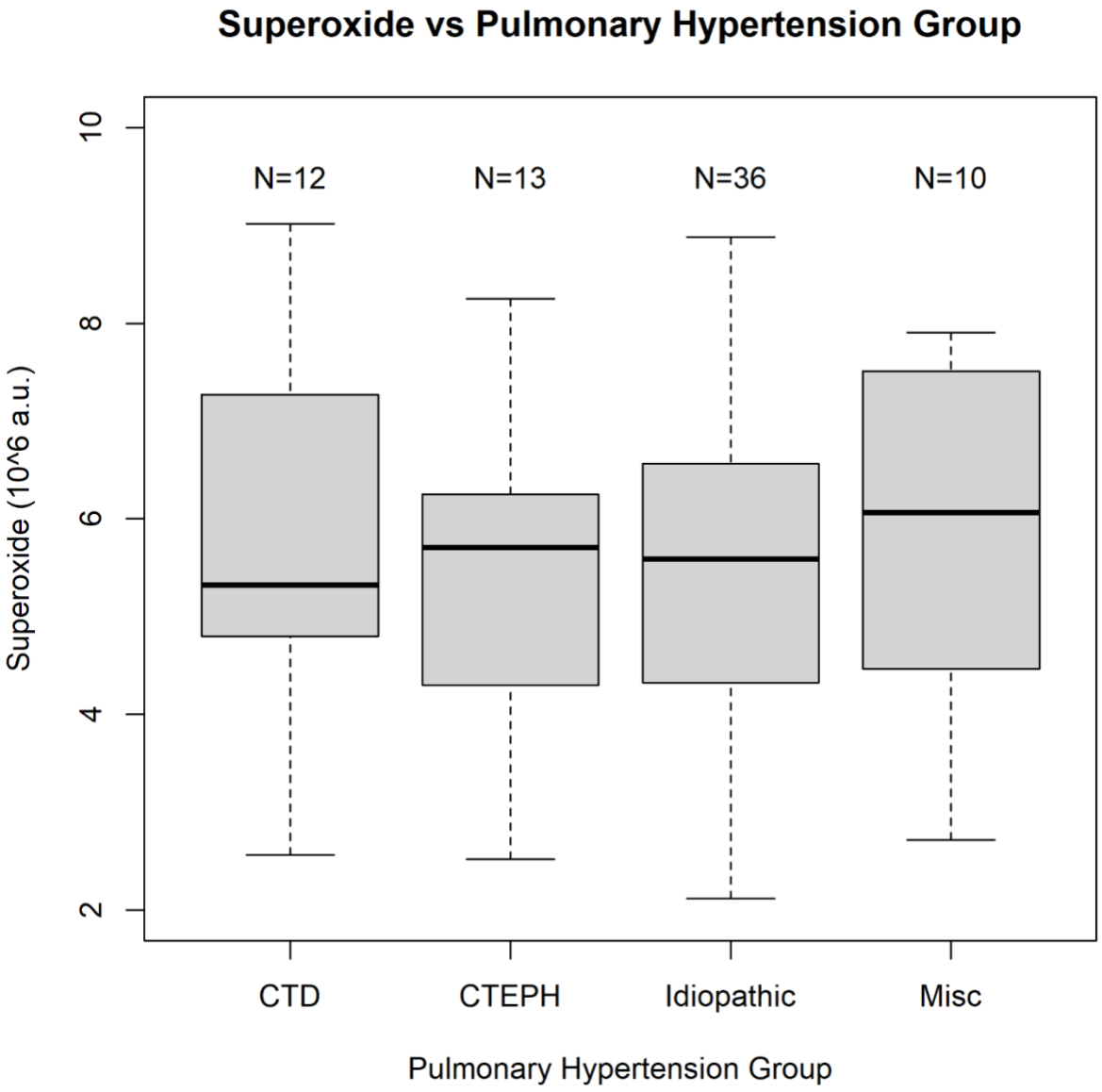


Figure 9: This boxplot shows $O_2^{\bullet-}$ levels versus the most common types of PH with no significant difference between groups. Misc. includes the patients with familial, portopulmonary and medication related PAH.

Due to the skewness of $O_2^{\bullet-}$ exhibited in the connective tissue disease (CTD) and CTEPH groups and the large imbalance between Idiopathic and the other three groups, the Kruskal Wallis test was used to determine evidence of differences in $O_2^{\bullet-}$ levels between groups. The Kruskal-Wallis chi-squared statistic was 0.60 with 3 degrees of freedom (p-

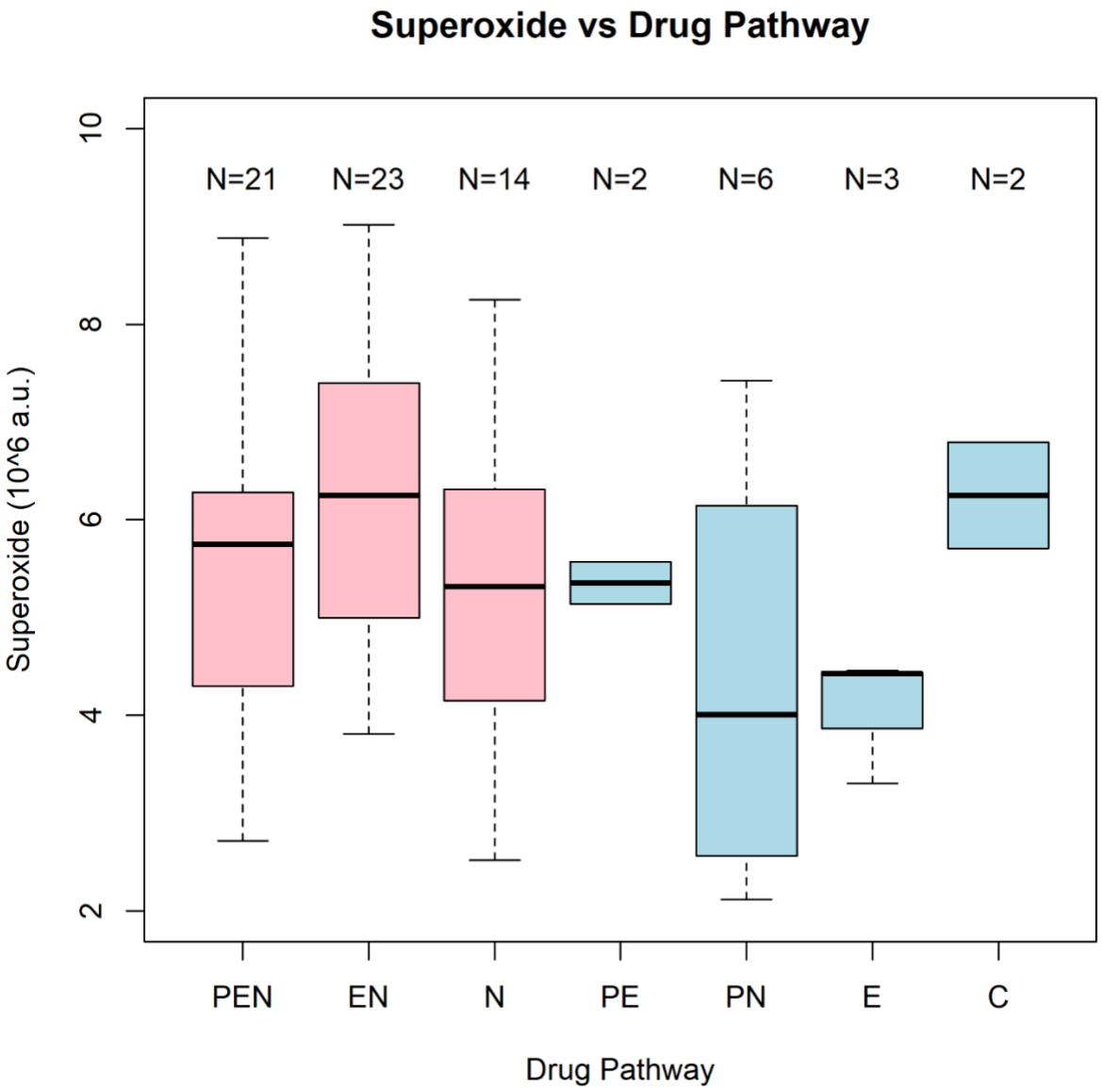
value = 0.8968) indicating that the median values of $O_2^{\bullet-}$ per group are not statistically distinguishable.

Table 10: Superoxide by PH etiology

PH Group	N	Median $O_2^{\bullet-}$ (a.u.)	IQR (Q1 – Q3)
CTD	12	5.3	4.8-7.3
CTEPH	13	5.7	4.3-6.3
Idiopathic	36	5.6	4.3-6.5
Miscellaneous	10	6.1	4.6-7.3

The cohort in this study was predominantly prevalent PAH patients, who were on a number of PH and non-PH medications, which may have confounded our results. However, when we stratified patients by type of PAH therapy, there was no difference in $O_2^{\bullet-}$ levels as seen in **Table 11** and **Figure 10**.

Figure 10: Superoxide levels and PAH Medication Type (Drug Pathway)



P = Prostacyclin pathway (prostacyclin or receptor agonist) E = Endothelin receptor antagonist pathway N = Nitric oxide pathway (phosphodiesterase 5 inhibitor or guanylate cyclase stimulator) C= calcium channel blocker

Figure 10: This boxplot shows $O_2^{\bullet-}$ levels by PAH treatment regimen. The boxes in light red indicate sample sizes that are large enough to make variance estimates, those in blue are too small for reliable variance estimates to be made.

Due to the skewness of $O_2^{\bullet-}$ exhibited in the ERA group and the small sample sizes in PE, PN, E and C drug pathway groups, the Kruskal Wallis test was used to determine evidence of differences in $O_2^{\bullet-}$ levels between groups. The Kruskal-Wallis chi-squared statistic was 9.25 with 6 degrees of freedom (p-value = 0.16) indicating that the median values of $O_2^{\bullet-}$ per group are not statistically distinguishable.

Table 11: Superoxide levels and PAH treatment type

Drug Pathway	N	Median $O_2^{\bullet-}$ ($x10^6$ a.u.)	IQR (Q1 – Q3)
PEN	21	5.7	4.3-6.3
EN	23	6.3	5.0-7.4
N	14	5.3	4.2-6.3
PE	2	5.4	5.2-5.5
PN	6	4.0	2.8-5.7
E	3	4.4	3.9-4.4
C	2	6.2	6.0-6.5

P = Prostacyclin pathway (prostacyclin or receptor agonist) E = Endothelin receptor antagonist pathway N = Nitric oxide pathway (phosphodiesterase 5 inhibitor or guanylate cyclase stimulator) C= calcium channel blocker

We also assessed whether sex had an impact on our results and there was no impact on $O_2^{\bullet-}$ levels as seen in **Table 12** and **Figure 11**.

Table 12: Superoxide levels and sex of subjects

Gender	N	Median	IQR (Q1 – Q3)
Male	23	6.1	4.5-6.8
Female	48	5.6	4.4-6.3

Due to the skewness of $O_2^{\bullet-}$ exhibited in males and for consistency, the Kruskal Wallis test was used to determine evidence of differences in $O_2^{\bullet-}$ levels between groups. The Kruskal-Wallis chi-squared statistic was 0.74 with 1 degree of freedom (p-value = 0.39) indicating that the median values of $O_2^{\bullet-}$ by sex are not statistically distinguishable.

Figure 11: Superoxide levels and sex of subjects

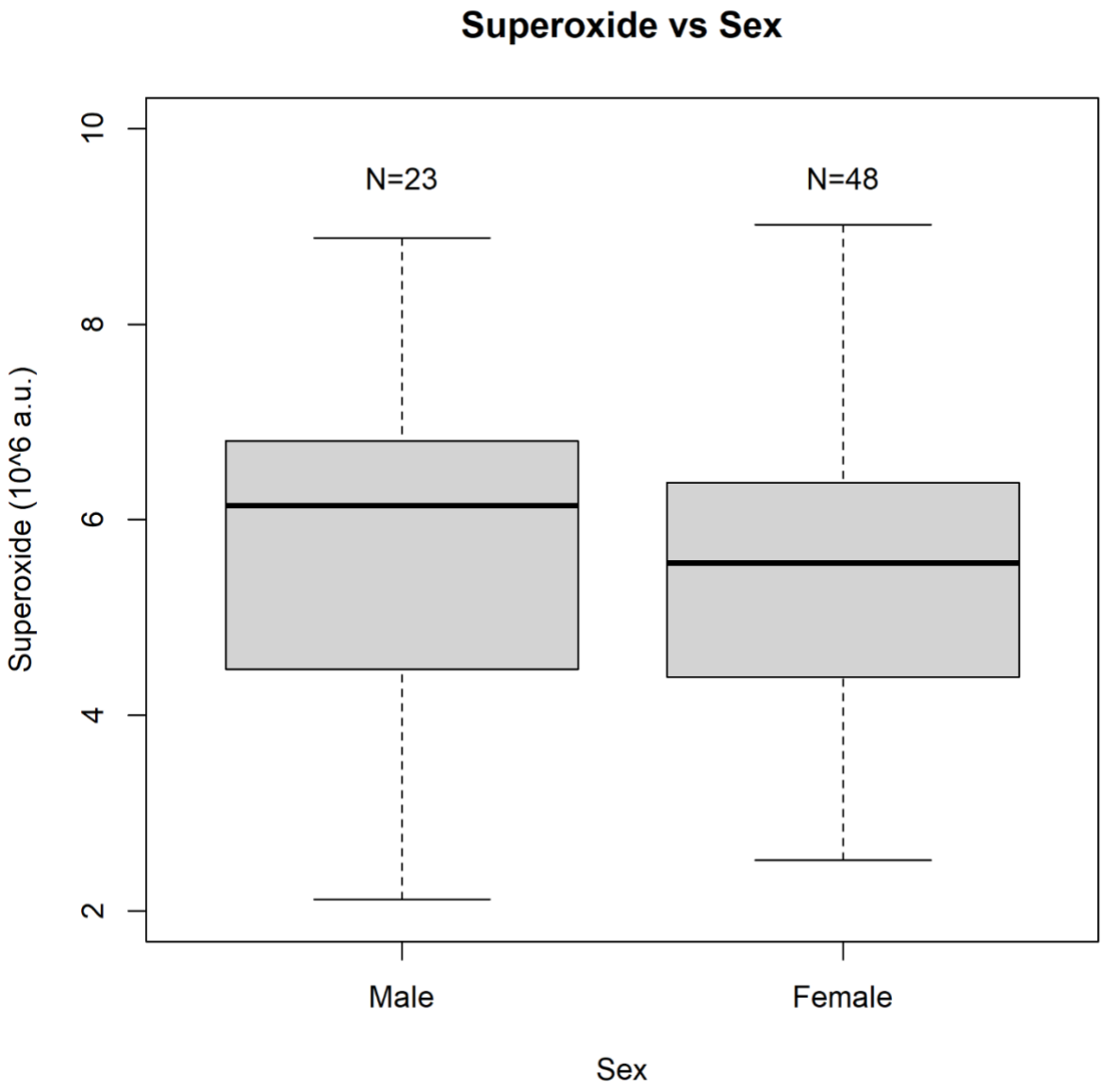


Figure 11: This boxplot shows $O_2^{\bullet-}$ levels by sex with no significant difference.

CHAPTER 4 DISCUSSION:

Superoxide is a byproduct of several enzymatic reactions, considered to be an intracellular messenger with a very short half-life. It can be a marker of oxidative stress and has been elevated in animal models of PH. This study is innovative because we utilized EPR Spectroscopy to measure $O_2^{\bullet-}$ levels in whole blood from PAH patients and carefully selected controls. Our pilot study data showed a significant correlation between $O_2^{\bullet-}$ levels in the pulmonary artery and peripheral blood. This was an important correlation because it provided rationale to use peripheral venous $O_2^{\bullet-}$ levels in a subsequent study. In the pilot study, levels of $O_2^{\bullet-}$ were elevated compared to normal subjects in a previous study in our lab.

However, when we subsequently did a case control study with carefully selected healthy controls with minimal medical comorbidities and with normal 6MW and echocardiograms, we were unable to demonstrate a significant difference in $O_2^{\bullet-}$ levels between patients with PH and controls. Our hypothesis was that patients with worse PH would have higher $O_2^{\bullet-}$ levels but levels of $O_2^{\bullet-}$ did not correlate with clinical measures of severity of PH including hemodynamic variables such as PVR and PAC, blood tests such as MVO_2 sats or BNP, functional tests such as 6MW or risk scores including WHO and REVEAL. It may be that $O_2^{\bullet-}$ levels are abnormal throughout the course of the pulmonary vascular dysfunction but we were unable to demonstrate an association with hemodynamic severity. It may be that oxidative stress is pathogenic in initiating pulmonary vascular remodeling as seen in animal models, but $O_2^{\bullet-}$ levels did not correlate with severity of hemodynamic compromise in our study. This is in contrast to other studies evaluating oxidative stress and PH. In a subset of 27 patients undergoing cardiac surgery, total antioxidant status correlated with PH diagnosed by echocardiography[117]. Notably, total antioxidant status is very much a different assessment than measuring $O_2^{\bullet-}$ levels with

EPR spectroscopy and our study measured $O_2^{\bullet-}$ levels directly in whole blood from patients with PAH confirmed by RHC. We did not find a significant association between $O_2^{\bullet-}$ and PAH, but many variables were not controlled including duration of disease, other medical comorbidities, physical activity, and medication use, all of which may impact $O_2^{\bullet-}$ levels.

Despite the specificity of EPR, which is considered by many to be the gold standard in measuring free radicals, like $O_2^{\bullet-}$, we were unable to detect a difference in $O_2^{\bullet-}$ levels between patients with PAH and healthy, control individuals. There are multiple reasons that our controls may have differed from the historical normal subjects from a prior study in our lab. Many variables including diet, alcohol intake, and exercise were not controlled for in this study and could serve to explain the higher variability in superoxide that was observed in controls compared to PAH cases. It is possible that the groups differed in antioxidant consumption or activity level. Exercise causes physiological stress as it increases oxygen consumption. In monocrotaline rats who underwent aerobic training, $O_2^{\bullet-}$ levels were increased compared to sedentary rats.[118] Our controls were normal healthy adults and exercised so this may have altered their $O_2^{\bullet-}$ levels compared to our historical controls. We did not limit exercise prior to blood draw in our control subjects and many walked a distance to get to our lab prior to having their blood drawn, which may have affected their $O_2^{\bullet-}$ levels. In contrast, our PAH patients are more sedentary, were often wheeled into clinic, and were sitting for varying lengths of time before their blood was drawn. We did not specifically address diet as it is extremely complex to assess dietary antioxidants in humans.[119]

Several other potential confounding variables were explored. We evaluated age as the patients with PAH were older than our healthy subjects. The association of $O_2^{\bullet-}$ and aging is incompletely understood. Early studies provided a basis for a free radical theory of aging associated with the damaging roles of oxidants including mitochondrial dysfunction, protein denaturation, altered cell membranes, cell death and senescence.[120]

However, redox signaling has also been found to be important in adaptive homeostasis.[121] Aging in rat pulmonary arteries is associated with impaired relaxation and oxidative stress assessed by dihydroethidine and chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester fluorescence assays.[122] In a study of blood samples from 500 patients undergoing cardiac surgery, total antioxidant status was measured and there was no correlation with age.[117] In that study, a fluorescent assay was used that assessed the ability of antioxidants to prevent oxidation of 2,2'-azino-di(3-ethylbenzothiazoline sulfonate. Interestingly, our study shows that in patients with PAH, $O_2^{\bullet-}$ levels decreased with age whereas in this small cohort of healthy subjects, $O_2^{\bullet-}$ levels increased with age. It is possible that adaptive homeostasis to oxidative stress with PAH may be beneficial and contribute to longevity in some patients with PAH.

Differences in BMI may also be a confounding variable in this study as the patients with PAH had a higher BMI than the control subjects. In an observational study that measured oxidative stress indices in obese males, they found higher levels of 8-iso-prostaglandin F2α, MDA, and lower levels of SOD in obese males suggesting increased oxidative stress.[123] Obesity was also associated with increased lipid peroxidation in another study as shown by increased levels of MDA and urinary F2-isoprostanes.[124] In the present study, $O_2^{\bullet-}$ showed no significant interaction with BMI in PAH patients or in healthy subjects so BMI is unlikely to be a confounding factor in our results.

Disease type, therapy, and disease duration also may be confounding variables in our results. This study included predominantly prevalent patients with PAH, and it is possible that the role of $O_2^{\bullet-}$ in the pathogenesis may evolve as the disease progresses. Many of our patients are on supplemental oxygen which may also affect $O_2^{\bullet-}$ levels. Although we included only Group 1 and Group 4 PAH, many etiologies are associated with Group 1 PAH and $O_2^{\bullet-}$ levels may differ depending on the specific underlying etiology. We found no difference between $O_2^{\bullet-}$ levels and the various etiologies of PAH in this cohort.

The effect of medications on $O_2^{\bullet-}$ levels is not clear and the PAH patients are often on multiple medications. We found no correlation between PAH medication treatment type and $O_2^{\bullet-}$ levels. Even though both groups were similar in terms of sex distribution, we assessed whether sex was a confounding factor but there was no difference in $O_2^{\bullet-}$ levels between males or females.

In conclusion, current evidence in animal models, ex-vivo PASMCs and PAECs and non-specific measures of oxidative stress *in vivo* suggest that ROS, including $O_2^{\bullet-}$, contribute to PAH by influencing pulmonary vascular remodeling and right ventricular hypertrophy. We hypothesized, like others,[125] that in PAH, oxidant stress dysregulates the normal compartmentalization of ROS signaling, leading to unrestrained positive feedback that contributes to the disease process, and that specifically measuring $O_2^{\bullet-}$ in peripheral blood would yield insight into the role of oxidative stress in PAH patients. Our results, however, do not support this hypothesis. Thus, it is likely that the oxidative stress noted in animal models of PH is too complex to be detected in humans with PAH by measuring $O_2^{\bullet-}$ as a single variable. Due to the multitude of effects of $O_2^{\bullet-}$ on lipid peroxidation, enzyme regulation, nucleotide oxygenation and metabolism, further studies may need to focus on the downstream effects of $O_2^{\bullet-}$, rather than on this specific cellular messenger.

PAH is a multifactorial disease characterized by the excessive proliferation of PASMCs and PAECs and extracellular matrix remodeling which may be triggered by many cellular, molecular, and genetic events. The abnormal vascular remodeling associated with PAH culminates in vasoconstriction and obstruction of pulmonary arteries, contributing to increased PVR, PAH, and RV failure. Despite advances in PAH therapy, the disease remains disabling and increases mortality. The complex molecular mechanisms involved in the pathobiology of PAH are limiting factors in the development of potential therapeutic interventions for PAH. It is likely that because PAH and oxidative stress are both multifactorial we were unable to detect a clinical correlation of PAH with

$O_2^{\bullet-}$ in this study. While multiple redox-associated processes appear to result in PAH, much remains to be elucidated regarding how these processes impact the progression, expression and treatment of PAH. Although we did not find an association between $O_2^{\bullet-}$ and PAH in humans, it is possible that other ROS and antioxidant levels/activity are altered in PAH. Further study of the molecular mechanisms of the effects of ROS on PH pathogenesis in humans is needed to further the development of therapeutic strategies for this devastating disease.

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APPENDIX A: Pilot Study Consent Form

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 598-17-EP **Page 1 of 7**

ADULT CONSENT - CLINICAL BIOMEDICAL
PAH ROS study consent form

Title of this Research Study
Assessment of oxidative stress at the time right heart catheterization in the work-up of patients suspected to have pulmonary arterial hypertension

Invitation
You are invited to take part in this research study. You have a copy of the following, which is meant to help you decide whether or not to take part:

- Informed consent form
- "What Do I need to Know Before Being in a Research Study?"
- The Rights of Research Subjects

Why are you being asked to be in this research study?
You are being asked to be in this study because you are older than 18 years-old, have pulmonary arterial hypertension, and are already clinically indicated for right heart catheterization. 22 subjects will be enrolled for this study at Nebraska Medicine. If you are pregnant you may not be in this study.

What is the reason for doing this research study?
Pulmonary arterial hypertension (PAH) is a poorly understood disease that can significant limit a patients functional status, reduced their quality of life, and decreased their life expectancy. The cause and processes leading to the progression of the disease is poorly understood, but research into some of these pathways have lead to some of the newer treatments of PAH. Still, despite many of our new advances in medical therapy, long-term survival remains poor for some with this disease. One pathway of disease process that has been suggested involves oxidative stress as related to PAH, however, to date these levels have not been directly measured in from the lungs and correlated to disease activity in humans. This study will correlate levels of oxidative stress in the lung blood vessels with disease activity and other blood markers to help better understand the process of PAH and possibly find future markers for disease prognosis and new potential targets for treatment.

What will be done during this research study?
There are no additional visits or costs associated with you participating in this study. After receiving your permission, the study team will collect health information such as your Name and medical records number for purpose of following up with you in the

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Valid until 10/23/2018

future. The study team will also collect results from your Six minute walk tests, NYHA functional class, and lab values such as your Congestive heart failure peptide levels, complete blood count, basic metabolic panel, and uric acid. Information from your Right Heart Catheterization, pulmonary function tests and echocardiograms will be entered into a secured, password protected database called RedCap, where you will be assigned a unique ID number.

A right heart catheterization (RHC) is typically used by your physician to diagnose or monitor PAH. Before your right heart catherization, a small amount of blood will be taken from a peripheral vein in your arm, similar to blood draw for routine labs. During the RHC, a small amount of blood, approximately 1 tsp, will be taken from your pulmonary artery. Taking blood from your pulmonary artery does not require any additional procedures or instruments and you will not feel anything.

What are the possible risks of being in this research study?
Blood draw: There is a slight risk of pain, bruises, or infections (very rarely) when blood is drawn for laboratory blood tests.

Blood draw from Pulmonary artery during Right-heart Catheterization: There is a slight risk (very rare) of developing anemia (low blood count).

Non-medical risks: The Non-medical risks involved in this study is the disclosure of your Protected Health Information (PHI). PHI is any health information that is collected about you, including your medical history and information collected through participating in this study. Although it would be very unlikely, the possible loss of the privacy of your PHI could happen. If the privacy of your PHI was lost, it could cause you stress, anxiety, embarrassment or other problems that could come from people or organizations
knowing your PHI who you don't want to have this information.

It is possible that other rare side effects could occur which are not described in this consent form. It is also possible that you could have a side effect that has not occurred before.

What are the possible benefits to you?
You are not expected to get any benefit from being in this research study.

What are the possible benefits to other people?
The results of this study may lead to better understanding and advancement of current medical knowledge of a currently poorly understood disease, pulmonary

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MR#:

CONSENT FORM

arterial hypertension. It may also lead to the development of future markers of disease progression that may help with future prognoses, as well as help define potential targets for treatment and disease modification.

What are the alternatives to being in this research study?
Instead of being in this research study, you can choose not to participate.

What will being in this research study cost you?
There is no cost to you to be in this research study.

Will you be paid for being in this research study?
You will not be paid to be in this research study.

Who is paying for this research?
This research is being paid for by the Department of Internal Medicine, Section of Pulmonary, Critical Care, Sleep & Allergy, University of Nebraska Foundation Kuhl Testamentary Trust Fund of the University of Nebraska Medical Center.

What should you do if you are injured or have a medical problem during this research study?
Your welfare is the main concern of every member of the research team. If you are injured or have a medical problem or some other kind of problem as a direct result of being in this study, you should immediately contact one of the people listed at the end of this consent form.

How will information about you be protected?
You have rights regarding the protection and privacy of your medical information collected before and during this research. This medical information is called "protected health information" (PHI). PHI used in this study may include your medical record number, address, birth date, medical history, the results of physical exams, blood tests, x-rays as well as the results of other diagnostic medical or research procedures. Only the minimum amount of PHI will be collected for this research. Your research and medical records will be maintained in a secure manner, over a secure encrypted server.

Who will have access to information about you?
By signing this consent form, you are allowing the research team to have access to your PHI. The research team includes the investigators listed on this consent form and other personnel involved in this specific study at the Institution.

IRB Approved
Valid until 10/23/2018

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 598-17-EP **Page 4 of 7**

Your PHI will be used only for the purpose(s) described in the section What is the reason for doing this research study?

You are also allowing the research team to share your PHI, as necessary, with other people or groups listed below:

- The UNMC Institutional Review Board (IRB)
- Institutional officials designated by the UNMC IRB
- Federal law requires that your information may be shared with these groups:
    - The HHS Office of Human Research Protections (OHRP)
- The HIPAA Privacy Rule requires the following groups to protect your PHI:
    - Researchers at UNMC involved in the study.

You are authorizing us to use and disclose your PHI for as long as the research study is being conducted.

You may cancel your authorization for further collection of PHI for use in this research at any time by contacting the principal investigator in writing. However, the PHI which is included in the research data obtained to date may still be used. If you cancel this authorization, you will no longer be able to participate in this research.

How will results of the research be made available to you during and after the study is finished?

In most cases, the results of the research can be made available to you when the study is completed, and all the results are analyzed by the investigator or the sponsor of the research. The information from this study may be published in scientific journals or presented at scientific meetings, but your identity will be kept strictly confidential.

If you want the results of the study, contact the Principal Investigator at the phone number given at the end of this form or by writing to the Principal Investigator at the following address:
Dr. Tammy Wichman
985990 Nebraska Medical Center
Omaha, NE 68198-5990

What will happen if you decide not to be in this research study?

You can decide not to be in this research study. Deciding not to be in this research will not affect your medical care or your relationship with the investigator or the Institution. Your investigator will still take care of you and you will not lose any benefits to which you are entitled.

IRB Approved
Valid until 10/23/2018

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 598-17-EP **Page 5 of 7**

What will happen if you decide to stop participating once you start?
You can stop participating in this research (withdraw) at any time by contacting the Principal Investigator or any of the research staff. Deciding to withdraw will otherwise not affect your care or your relationship with the investigator or this institution. You will not lose any benefits to which you are entitled. Any research data obtained to date may still be used in the research.

Will you be given any important information during the study?
You will be informed promptly if the research team gets any new information during this research study that may affect whether you would want to continue being in the study, however this is very unlikely given the nature of this study.

What should you do if you have any questions about the study?
You have been given a copy of "*What Do I Need to Know Before Being in a Research Study?*" If you have any questions at any time about this study, you should contact the Principal Investigator or any of the study personnel listed on this consent form or any other documents that you have been given.

What are your rights as a research participant?
You have rights as a research subject. These rights have been explained in this consent form and in The Rights of Research Subjects that you have been given. If you have any questions concerning your rights, or want to discuss problems, concerns, obtain information or offer input, or make a complaint about the research, you can contact any of the following:

- The investigator or other study personnel
- Institutional Review Board (IRB)
  - Telephone: (402) 559-6463.
  - Email: IRBORA@unmc.edu
  - Mail: UNMC Institutional Review Board, 987830 Nebraska Medical Center, Omaha, NE 68198-7830
- Research Subject Advocate
  - Telephone: (402) 559-6941
  - Email: unmcrsa@unmc.edu

Documentation of informed consent
You are freely making a decision whether to be in this research study. Signing this form means that:

- You have read and understood this consent form.
- You have had the consent form explained to you.

IRB Approved
Valid until 10/23/2018

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 598-17-EP **Page 6 of 7**

- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.
- You have decided to be in the research study.
- If you have any questions during the study, you have been directed to talk to one of the investigators listed below on this consent form.
- You will be given a signed and dated copy of this consent form to keep.

Signature of Subject _____

Date _____

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the subject. In my judgment, the subject possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate.

Signature of Person obtaining consent _____

Date _____

Authorized Study Personnel
Principal
* Wichman, Tammy
phone: 402-559-9472
alt #: 402-559-9472
degree: M.D.

Secondary
* Sklare, Seth
phone: 402-559-7268
alt #: 402-559-6315
degree: MD

Data/Administrative Personnel
Ahmad, Iman
phone: 402-559-6911
alt #: 402-559-6911

Zimmerman, Matthew
phone: 402-559-7842
alt #: 402-559-7842

IRB Approved
Valid until 10/23/2018

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 598-17-EP **Page 7 of 7**

degree: PhD

degree: PhD

Participating Personnel

Duryee, Michael
phone: 402-559-7030
alt #: 402-559-7030
degree: MS

Hamilton, Bartlett
phone: 402-559-9036
alt #: 402-559-9036
degree: BA

* Zolty, Ronald
phone: 402-559-5151
alt #: 402-559-4224
degree: MD

Lead Coordinator

Nelson, Traci
phone: 402-559-7585
alt #: 402-559-7585
degree: M.S.

IRB Approved
Valid until 10/23/2018

APPENDIX B: Controls Consent Form

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 1 of 6**

ADULT CONSENT - REGISTRY
PAH Registry controls

Title of this Registry
Pulmonary Arterial Hypertension Registry

Invitation
You are invited to take part in this registry. You have a copy of the following, which is meant to help you decide whether or not to take part:

- Informed consent form
- "What Do I need to Know Before Being in a Research Study?"
- The Rights of Research Subjects

Why are you being asked to be in this registry?
You are being asked to be in this registry because you are 19 years of age or older and have no clinical evidence of heart or lung disease.

What is the reason for doing this registry?
We will collect data to compare people who do not have pulmonary hypertension to those affected with the disease.

What will be done during this registry?
An investigator will obtain a history and perform a brief physical exam.

You will fill out questionnaires about the quality of your life.

Approximately 4 teaspoons of blood will be drawn from a vein in your arm.

You will walk for six minutes with a respiratory therapist recording oxygen levels and distance.

You will have an echocardiogram done which involves using gel and an ultrasound machine to obtain pictures of your heart.

What are the possible risks of being in this registry?
There is a risk of bleeding and bruising at the site blood is drawn.

You may have a loss of confidentiality if unauthorized individuals gain access to your information.

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Valid until 09/03/2021

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 2 of 6**

What are the possible benefits to you?
You are not expected to get any benefit from being in this registry

What are the possible benefits to other people?
The knowledge gained from this registry may benefit doctors and investigators interested in treating and understanding pulmonary hypertension.

What are the alternatives to being in this registry?
Instead of being in this registry, you can choose not to participate.

What will being in this registry cost you?
There is no cost to you to be in this registry.

Will you be paid for being in this registry?
You will be paid $100 to be in this registry.

In the event that any commercial products are developed from your donated tissue, there are no plans to share any revenues with you from those commercial products. It is policy that all donated tissue is the property of the Organization.

Who is paying for this registry?
This registry is being paid for by pilot grant funding from the Clinical Research Center at the University of Nebraska Medical Center.

What should you do if you have a problem during this registry?
Your welfare is the main concern of every member of the registry team. If you have a problem as a direct result of being in this registry, you should immediately contact one of the people listed at the end of this consent form.

How will information about you be protected?
You have rights regarding the protection and privacy of your medical information collected for this registry. This medical information is called "protected health information" (PHI). PHI used in this registry may include your medical record number, address, birth date, medical history, the results of physical exams, blood tests, x-rays, as well as the results of other diagnostic and medical procedures, as well as your medical history. Your data and medical records will be maintained in a secure manner.

Who will have access to information about you?
By signing this consent form, you are allowing the investigators listed on the consent

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form to have access to your PHI. The registry team includes the investigators listed on this consent form and other personnel involved in this specific registry at the Institution.

Your PHI will be used only for the purpose(s) described in the section "What is the reason for doing this registry?"

You are also allowing the registry team to share your PHI, as necessary, with other people or groups listed below:

- The UNMC Institutional Review Board (IRB)
- Institutional officials designated by the UNMC

You are authorizing us to use and disclose your PHI for as long as the registry is being conducted.

You may cancel your authorization for further collection of PHI for use in this registry at any time by contacting the principal investigator in writing. However, the PHI which is included in the registry data obtained to date may still be used. If you cancel this authorization, you will no longer be able to participate in this registry.

What will happen if you decide not to be in this registry?
You can decide not to be in this registry. Deciding not to be in this registry will not affect your relationship with the registry team or the Institution.

You will not lose any benefits to which you are entitled.

What will happen if you decide to stop participating once you start?
You can stop participating in this registry ("withdraw") at any time. Should you wish to stop participating in the registry, you must contact one of these individuals in writing requesting that your data no longer be used for future research purposes.

Tammy Wichman MD
985910 Nebraska Medical Center
Omana NE 68198-5910

Deciding to withdraw will not otherwise affect your relationship with the registry team or this institution. You will not lose any benefits to which you are entitled.

Data kept without identifiers are unable to be withdrawn.

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 4 of 6**

Will you be given any important information during the registry?
If the registry team gets any new information during this registry that may affect whether you would want to continue being in the registry, you will be informed promptly.

What should you do if you have any questions about the registry?
You have been given a copy of "What Do I Need to Know Before Being in a Research Study?" If you have any questions at any time about this registry, you should contact the Principal Investigator or any of the registry personnel listed on this consent form or any other documents that you have been given.

What are your rights as a registry participant?
You have rights as a registry participant. These rights have been explained in this consent form and in "The Rights of Research Subjects" that you have been given. If you have any questions concerning your rights or complaints about the registry, you can contact any of the following:

- The investigator or other registry personnel
- Institutional Review Board (IRB)
  - Telephone: (402) 559-6463.
  - Email: IRBORA@unmc.edu
  - Mail: UNMC Institutional Review Board, 987830 Nebraska Medical Center, Omaha, NE 68198-7830
- Research Subject Advocate
  - Telephone: (402) 559-6941.
  - Email: unmcrsa@unmc.edu

Documentation of informed consent
You are freely making a decision whether to participate in the registry. Signing this form means that:

- You have read and understood this consent form.
- You have had the consent form explained to you.
- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.
- You have decided to participate in the registry.
- If you have any questions, you have been directed to talk to one of the individuals listed below on this consent form.
- You will be given a signed and dated copy of this consent form to keep.

IRB Approved
Valid until 09/03/2021

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 5 of 6**

Signature of Participant _____
Date _____

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the participant, and all of their questions have been answered. In my judgment, the participant possesses the capacity to give informed consent to participate in this registry and is voluntarily and knowingly giving informed consent to participate.

Signature of Person obtaining consent _____
Date _____

Authorized Registry Personnel
Principal
* Wichman, Tammy
phone: 402-559-9472
alt #: 402-559-9472
degree: M.D.

Secondary
* Thompson, Austin
phone: 402-559-7537
alt #: 402-559-3777
degree: M.D.

Participating Personnel
Davidson, Ross
phone: 402-559-8115
alt #: 402-559-7288
degree: MD`

Lead Coordinator
Sweeter, Jenea
phone: 402-559-7585
alt #: 402-841-9266
degree: BS

Other Coordinator
Lane, Judy
phone: 402-559-7555

IRB Approved
Valid until 09/03/2021

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 6 of 6**

alt #: 402-559-3777
degree: R.N.

APPENDIX C: PAH Patient Consent Form

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 1 of 7**

ADULT CONSENT - REGISTRY
PAH Registry

Title of this Registry
Pulmonary Arterial Hypertension Registry

Invitation
You are invited to take part in this registry. You have a copy of the following, which is meant to help you decide whether or not to take part:

- Informed consent form
- "What Do I need to Know Before Being in a Research Study?"
- The Rights of Research Subjects

Why are you being asked to be in this registry?
You are being asked to be in this registry because you are 19 years of age or older and have been diagnosed with Pulmonary Arterial Hypertension (PAH).

What is the reason for doing this registry?
The purpose of this project is to establish a registry (database) of patients with PAH. The registry will be used to follow clinical outcomes, to provide a database from which to recruit subjects for clinical investigations and to provide clinical information for research and to provide optimal treatment options based on data collected.

The project will also collect serum/blood samples to be banked for any future studies. Blood samples will be collected in addition to other physician ordered labs.

What will be done during this registry?
There are no additional visits or costs associated with you participating in this study. After receiving your permission, the study team will enter elements of your medical history and your pulmonary hypertension into the database. Listed below is a description of the data to be collected for the registry:

- Name
- Address
- Phone number
- Height
- Weight
- Gender
- Race
- Pregnancy/Live birth(s)

- Diagnosis
- Referring physician
- Vitals
- All labs and pathology results
- All diagnostic tests (i.e. X-ray, CT, PFT, etc.)
- All procedures (i.e. cath lab, vascular lab, etc.)
- Medications
- Hospitalizations and ER visits
- Survival/death
- Complications
- Nutrition
- Last clinic visit
- Acute exacerbations
- Transplantation
- Trial participation
- Flags (i.e. infection control precautions)

Optional Blood Samples
About 1-2 tablespoons (15-30 mL) of blood will be obtained from a vein in your arm and may be used for future testing to learn how to develop a good blood test for periostin or other blood proteins. If you withdraw from the study, we may still perform testing on your remaining blood.

As future studies become available, the database will be screened for patients that meet specific criteria. If you meet the search criteria, you may be contacted to determine if you are interested in participating in an investigational study.

What are the possible risks of being in this registry?
The non-medical risk involved in an information-gathering registry is the disclosure of your Protected Health Information (PHI). PHI is any health information that is collected about you, including your medical history and information collected through participating in this Study. This information is stored indefinitely.

Although it would be very unlikely, the possible loss of the privacy of your PHI could happen. If the privacy of your PHI was lost, it could cause you stress, anxiety, embarrassment or other problems that could come from people or organizations knowing your PHI who you don't want to have this information.

Risks of Blood Testing:
You may experience slight bruising, a lump, or pain on your arm where blood tests

are taken. There is also the slight risk of infection, light-headedness, and/or fainting.

What are the possible benefits to you?
You are not expected to get any benefit from being in this registry.

What are the possible benefits to other people?
The knowledge gained from this registry may contribute to information that would improve the treatment for you and other patients with PAH.

What are the alternatives to being in this registry?
Your participation in this registry is voluntary. If you do not participate in the study or if your participation is discontinued, your physician will continue to treat you with standard medical treatment.

What will being in this registry cost you?
There is no cost to you to be in this registry.

Will you be paid for being in this registry?
You will not be paid to be in this registry.

Who is paying for this registry?
This registry is being paid for by the Department of Internal Medicine, Section of Pulmonary.

What should you do if you have a problem during this registry?
Your welfare is the main concern of every member of the registry team. If you have a problem as a direct result of being in this registry, you should immediately contact one of the people listed at the end of this consent form.

How will information about you be protected?
You have rights regarding the protection and privacy of your medical information collected for this registry. This medical information is called "protected health information" (PHI). PHI used in this registry may include your medical record number, address, birth date, medical history, the results of physical exams, blood tests, x-rays, as well as the results of other diagnostic and medical procedures, as well as your medical history. Your data and medical records will be maintained in a secure manner.

Who will have access to information about you?
By signing this consent form, you are allowing the investigators listed on the consent

form to have access to your PHI. The registry team includes the investigators listed on this consent form and other personnel involved in this specific registry at the Institution.

Your PHI will be used only for the purpose(s) described in the section "What is the reason for doing this registry?"

You are also allowing the registry team to share your PHI, as necessary, with other people or groups listed below:

- The UNMC Institutional Review Board (IRB)
- Institutional officials designated by the UNMC IRB
- Federal law requires that your information may be shared with these groups:
    - The HHS Office of Human Research Protections (OHRP)
    - The Food and Drug Administration (FDA)

You are authorizing us to use and disclose your PHI for as long as the registry is being conducted.

You may cancel your authorization for further collection of PHI for use in this registry at any time by contacting the principal investigator in writing. However, the PHI which is included in the registry data obtained to date may still be used. If you cancel this authorization, you will no longer be able to participate in this registry.

What will happen if you decide not to be in this registry?
You can decide not to be in this registry. Deciding not to be in this registry will not affect your medical care or your relationship with the investigator or the Institution. Your doctor will still take care of you.

You will not lose any benefits to which you are entitled.

What will happen if you decide to stop participating once you start?
You can stop participating in this registry ("withdraw") at any time. Should you wish to stop participating in the registry, you must contact one of these individuals in writing requesting that your data no longer be used for future research purposes.

University of Nebraska Medical Center
Attn: Tammy Wichman, M.D.
985990 Nebraska Medical Center
Omaha, NE. 68198-5990

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

Deciding to withdraw will not otherwise affect your care or your relationship with the registry team or this institution. You will not lose any benefits to which you are entitled.

Data that is obtained and kept without identifiers are unable to be withdrawn.

Will you be given any important information during the registry?
If the registry team gets any new information during this registry that may affect whether you would want to continue being in the registry, you will be informed promptly.

What should you do if you have any questions about the registry?
You have been given a copy of "What Do I Need to Know Before Being in a Research Study?" If you have any questions at any time about this registry, you should contact the Principal Investigator or any of the registry personnel listed on this consent form or any other documents that you have been given.

What are your rights as a registry participant?
You have rights as a registry participant. These rights have been explained in this consent form and in The Rights of Research Subjects that you have been given. If you have any questions concerning your rights or complaints about the registry, you can contact any of the following:

- The investigator or other registry personnel
- Institutional Review Board (IRB)
  - Telephone: (402) 559-6463.
  - Email: IRBORA@unmc.edu
  - Mail: UNMC Institutional Review Board, 987830 Nebraska Medical Center, Omaha, NE 68198-7830
- Research Subject Advocate
  - Telephone: (402) 559-6941.
  - Email: unmcrsa@unmc.edu

Documentation of informed consent
You are freely making a decision whether to participate in the registry. Signing this form means that:

- You have read and understood this consent form.
- You have had the consent form explained to you.
- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 6 of 7**

- You have decided to participate in the registry.
- If you have any questions, you have been directed to talk to one of the individuals listed below on this consent form.
- You will be given a signed and dated copy of this consent form to keep.

Signature of Participant _____

Date _____

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the participant, and all of their questions have been answered. In my judgment, the participant possesses the capacity to give informed consent to participate in this registry and is voluntarily and knowingly giving informed consent to participate.

Signature of Person obtaining consent _____

Date _____

Optional Blood Collection for Biorepository:
By initialing below, you indicate your wish to participate in the optional blood collection. The blood samples collected, as a part of this optional testing, can be used for the purposes of future research and any property rights you may have to the samples collected for this optional research are revoked. You understand that you do not have to participate in this optional blood collection in order to participate in this registry.

_____ (initials) Yes, you will participate in the optional blood collection for use in future research.

_____ (initials) No, you will not participate in the optional blood collection for use in future research.

Authorized Registry Personnel
Principal
* Wichman, Tammy
phone: 402-559-9472
alt #: 402-559-9472

IRB
Approved 09/03/2020
Valid until 09/03/2021

Nebraska Medicine
SERIOUS MEDICINE. EXTRAORDINARY CARE.

PT NAME:
MR#:

CONSENT FORM

IRB PROTOCOL # 786-16-EP **Page 7 of 7**

degree: M.D.

Secondary

Davidson, Ross
phone: 402-559-8115
alt #: 402-559-7288
degree: MD`

* Thompson, Austin
phone: 402-559-7537
alt #: 402-559-3777
degree: M.D.

Participating Personnel

Duryee, Michael
phone: 402-559-7030
alt #: 402-559-7030
degree: MS

Hamilton, Bartlett
phone: 402-559-9036
alt #: 402-559-9036
degree: BA

Thiele, Geoffrey
phone: 402-559-7010
alt #: 402-559-8720
degree: PhD

Lead Coordinator

Sweeter, Jenea
phone: 402-559-7585
alt #: 402-841-9266
degree: BS

Other Coordinator

Lane, Judy
phone: 402-559-7555
alt #: 402-559-3777
degree: R.N.