Is blood flow through IPAVA and PFO related to breath-hold and SCUBA diving-induced pulmonary hypertension?

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Research Plan

IMPORTANT: When completing this outline, please use the **Research Plan Guidance** for the content necessary to develop a comprehensive yet succinct Research Plan. Using the guidance to complete this outline will help facilitate timely IRB review.

Study Title: Is blood flow through IPAVA and PFO related to breath-hold and SCUBA diving-induced pulmonary hypertension?

Protocol Number: 07302018.031

Principal Investigator: Andrew T Lovering, PhD

A. Introduction and Background

Pulmonary arterial hypertension (increased lung blood pressure) is a multifactorial disease without a cure. Investigating reversible forms of pulmonary hypertension induced under extreme conditions such as a prolonged breath-hold and/or SCUBA diving may help to better understand why some individuals develop this devastating disease, and others do not.

Pulmonary arterial pressure is typically very low in healthy humans. Low oxygen levels (hypoxia) cause a constriction of the lung blood vessels resulting in smaller diameters, but lung blood flow stays constant or increases. Having constant flow with smaller vessel diameters causes pulmonary arterial pressure to increase but it will return to normal once normal oxygen levels are restored. Moreover, there is an association with patent foramen ovale (PFO, small hole between the atria in the heart) and excessive pulmonary arterial pressures in low oxygen conditions. However, the reasons for the exacerbated increase in pulmonary arterial pressure in these subjects with a PFO (PFO+) is unknown, but may be due to an exaggerated constriction response to low oxygen.

During a breath-hold, the oxygen in the lung decreases and in elite breath-hold divers, it decreases to very low levels. Compared to subjects without a PFO (PFO-), the oxygen may drop even lower in those PFO+ subjects because deoxygenated blood travels through the small hole to mix with oxygenated blood, exacerbating the level to which blood oxygen decreases. Thus, using a breath-hold model of lung hypoxia is one approach to examining a hypoxia-induced increase in pulmonary arterial pressure – a method critically dependent upon the elite breath-hold diver's ability to hold their breath for significant durations.

Intrapulmonary arteriovenous anastomoses (IPAVA) are vessels within the lung that bypass capillaries. My group has investigated the possible roles these unique vessels may have in physiological and pathophysiological conditions. We have found that IPAVA blood flow occurs when healthy subjects breathe low oxygen gas. We have also found that IPAVA blood flow is inversely related to pulmonary arterial pressure. Specifically, individuals with high pulmonary arterial pressures have low IPAVA blood flow and vice versa.

The right ventricle of the heart pumps blood through the pulmonary artery to the lungs. Under resting conditions the right heart performs a minimal amount of work because the pressure in the lung blood vessels is low. When pulmonary arterial pressure increases, the work of the right side of the heart has to increase substantially to keep blood pumping through the lung. Thus, high pulmonary arterial pressures will increase the work of the right heart and may lead to right heart dysfunction thereby limiting the amount of blood the heart can pump. If the pressure is high enough to limit the amount of blood flowing through the lung then this can be detected by a reduction in pulmonary blood flow and/or changes in the function of the heart during contraction

(systole) and relaxation (diastole). Accordingly, an intervention that reduces pulmonary arterial pressures during a breath-hold may have a beneficial effect on right heart function.

Taken together, during an elite breath-hold dive, where the level of oxygen decreases, we expect that pulmonary arterial pressures will increase as lung oxygen levels decrease, and the reduction in oxygen may be even lower in PFO+ subjects. Furthermore, as blood oxygen levels decrease, IPAVA blood flow will increase in some PFO- subjects thereby keeping pulmonary arterial pressures low in those individuals. Conversely, PFO+ subjects and those PFO- subjects with low levels of IPAVA blood flow would be expected to have the greatest pulmonary pressures. Whether or not this is true is unknown. Therefore, **Objective #1** will quantify pulmonary arterial pressure and right heart function and investigate their relationships with PFO and IPAVA blood flow in elite breath-hold divers while breathing concentrations of oxygen and carbon dioxide that mimic breath-hold-induced hypoxia [NOTE: it is not possible to image the heart during a breath hold because the fully inflated lung obstructs the ultrasound view of the heart]. **Objective #2** will use sildenafil, a drug that increases nitric oxide bioavailability to dilate lung blood vessels, to decrease pulmonary arterial pressure while breathing concentrations of oxygen and carbon dioxide that mimic breath-hold-induced hypoxia. We will quantify the effect of sildenafil on pulmonary arterial pressure and right heart function and will determine if it alters the relationship with IPAVA and PFO blood flow. As mentioned above, because those PFO+ subjects may have an exaggerated pulmonary vasoconstrictor response to hypoxia, sildenafil may be either less effective or ineffective in reducing the pulmonary arterial pressure in these subjects. **Objective #3** will compare the elite breath hold diver study data to data obtained in age, sex and PFO matched subjects who do not have extensive experience with breath hold diving. To do this control subjects will undergo the same procedures in **Objectives #1 & 2** above. These studies will also allow us to determine if there are differences in pulmonary vascular responses to hypoxia between those with and without breath hold diving experience.

In addition to the heart and lung alterations that occur in breath hold divers outlined above, it is also known that pulmonary arterial pressure increases after SCUBA diving, but returns to normal within a few hours. The mechanisms responsible for the increase in pulmonary arterial pressure are unknown, but are independent of hypoxia. Thus, investigating the relationship between IPAVA, PFO and SCUBA diving-induced increases in pulmonary arterial pressures offers an additional avenue for understanding pulmonary arterial hypertension susceptibility. Although it is unknown why pulmonary arterial pressure increases with SCUBA diving, it is known that pulmonary hypertension may contribute to right heart dysfunction and pulmonary edema (lung water accumulation) that can occur in subjects who are swimming and/or SCUBA diving. Prevention of increased pulmonary arterial pressures during and/or after a dive may help to prevent excessive right heart dysfunction and pulmonary edema. Thus, **Objective #4** will quantify pulmonary arterial pressure and right heart function and investigate their relationships with PFO and IPAVA blood flow, pre- and post-SCUBA diving. **Objective #5** will quantify the effect of sildenafil (post dive) on pulmonary arterial pressure and right heart function and will determine if it alters the relationship with IPAVA and PFO blood flow, pre- and post-SCUBA diving.

In summary, we propose to study elite Croatian breath-hold and SCUBA divers. We will quantify breath-hold hypoxia- and SCUBA diving-induced pulmonary hypertension and right heart function to investigate the relationships between PFO and IPAVA blood flow. We will use a placebo-controlled intervention (sildenafil) to

reduce pulmonary arterial pressure in these subjects to examine the impact of the change in pressure (or absence of change) on the relationships determined above.

B. Specific Aims/Study Objectives

The overarching goal of this study is to examine cardiopulmonary and respiratory physiology in breath hold and SCUBA divers.

In breath hold divers we will:

1) Quantify pulmonary arterial pressure and right heart function and determine their relationships with PFO and IPAVA blood flow in elite breath-hold divers. We **hypothesize**:
   a. PFO- subjects with high pulmonary arterial pressures will have low IPAVA blood flow, and vice versa.
   b. PFO+ subjects will have high pulmonary arterial pressures independent of blood flow through IPAVA.
   c. Subjects with the highest pulmonary arterial pressures will have the greatest right heart dysfunction, and vice versa.

2) Quantify the effect of sildenafil on pulmonary arterial pressure and right heart function and determine their relationships with PFO and IPAVA blood flow in elite breath-hold divers. We **hypothesize**:
   a. Sildenafil will reduce pulmonary arterial pressure, thereby reducing IPAVA blood flow in PFO- subjects.
   b. Sildenafil will be less effective/ineffective in reducing high pulmonary arterial pressures in PFO+ subjects, independent of IPAVA blood flow.
   c. Subjects with the highest pulmonary arterial pressures will have the greatest restoration of right heart function with sildenafil treatment, and vice versa.

3) Quantify pulmonary arterial pressure and right heart function and determine their relationships with PFO and IPAVA blood flow in age, sex and PFO matched control subjects. Also, quantify the effects of sildenafil on these measures in age, sex and PFO matched control subjects. We **hypothesize**:
   a. Control subjects will have a greater pulmonary pressure and lower IPAVA blood flow compared to age, sex and PFO status breath hold divers.
   b. Subjects with the highest pulmonary arterial pressures will have the greatest right heart dysfunction, and vice versa.
   c. Subjects with the highest pulmonary arterial pressures will have the greatest restoration of right heart function with sildenafil treatment, and vice versa.

In SCUBA divers we will:

4) Quantify pulmonary arterial pressure and right heart function and determine their relationships with PFO and IPAVA blood flow in SCUBA divers. We **hypothesize**:
   a. PFO- subjects with high pulmonary arterial pressures will have low IPAVA blood flow, and vice versa.
   b. PFO+ subjects will have high pulmonary arterial pressures independent of IPAVA blood flow.
   c. Subjects with the highest pulmonary arterial pressures will have the greatest right heart dysfunction, and vice versa.

5) Quantify the effect of sildenafil on pulmonary arterial pressure and right heart function and determine their relationships with PFO and IPAVA blood flow and in SCUBA divers. We **hypothesize**:
   a. Sildenafil will reduce pulmonary arterial pressures, thereby reducing IPAVA blood flow in PFO subjects.
   b. Sildenafil will be less effective/ineffective in reducing high pulmonary arterial pressures in PFO+ subjects.

c. Subjects with the highest pulmonary arterial pressures will have the greatest restoration of right heart function with sildenafil treatment.

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C. Methods, Materials and Analysis

This study will be performed over summers 2019 and 2022 in Split Croatia and the Cardiopulmonary and Respiratory Physiology lab at the University of Oregon in Eugene, Oregon for research activities involving breath hold diving and SCUBA diving subjects. We will perform research activities in breath hold divers and age and sex-matched breath hold diver controls while in Split Croatia. In Eugene, OR we will only perform research activities in age, PFO, and sex-matched breath hold diver controls. SCUBA diving studies will only be performed in Croatia. My lab has extensive experience examining IPAVA and PFO blood flow as well as measuring pulmonary artery pressure under various environmental challenges such as during exercise, pre- and post-diving and high altitude. We will use saline contrast echocardiography to detect and differentiate between blood flow through IPAVA and PFO, as we have many times before. We will use Doppler ultrasound as before to quantify differences in pulmonary artery pressure with and without the use of sildenafil. We will use the Journal of American Society for Echocardiography recommendations for making right and left heart function measures as before. We will collaborate with Professor Željko Dujić, MD, PhD who has extensive experience working with professional, elite, world-record level, breath-hold divers and SCUBA divers. He also has the required laboratory space and resources for performing these experiments at the University of Split Medical School, Split Croatia. There are many elite breath-hold divers that live in Croatia and Dr. Dujić has established an excellent rapport with the divers and their coach Ivan Drvis who is with the Faculty of Kinesiology at the University of Zagreb, Croatia. Additionally, Dr. Dujić has also established an excellent rapport with the Croatian Military for the SCUBA diving studies to be performed using highly experienced divers on a Croatian Navy Base field site that has the required laboratory space and resources for performing the proposed studies. Dr. Dujić has significant experience working with these groups so it will prevent the possibility of any cultural or political issues interfering with this work. Additionally, Dr. Dujić and I have worked together before on several projects, see Lovering CV for verification as needed.

Briefly we will use a single-blind, placebo-controlled study to determine the impact of sildenafil (50 mg, oral) on IPAVA and PFO blood flow and right heart function and pulmonary pressures. We will use 40 breath-hold divers (20 PFO+, 20 female), 40 age-matched (within 5 years) breath-hold diver Controls (20 PFO+, 20 female), and 50 (25 PFO+, 25 female) SCUBA divers. Sildenafil will be given before the breath hold in the breath-hold divers but after SCUBA diving to avoid any potential decompression illness complications.

Screening at each study visit: All subjects with child bearing potential will be required to take a urine pregnancy test before each visit begins. A positive test at any time will result in exclusion.

Breath Hold Diving Subjects & Controls (Objectives 1 - 3)

Visit 1 Overview: Subject consent, screening, lung function and anthropometrics. Subjects will ingest either a placebo or sildenafil (50mg, oral), wait an hour for peak effect and then breathe low oxygen and high CO2 gas mixtures that mimic blood gases during a breath hold, during which ultrasound measures of the heart and lungs will be made (~4.5 hrs). Note: Breath hold subjects will breathe lower oxygen concentrations and higher carbon dioxide levels than control subjects but slopes of these responses will still be comparable.

Visit 1 Details:
Subjects will undergo informed consent, fill out a health history questionnaire (~45 min).

Subjects will be comprehensively screened for any cardiac abnormalities. Amount of blood flow travelling through the PFO and IPAVA will be graded using saline contrast echocardiography while the subject is breathing

room air (PFO and IPAVA blood flow) and breathing 100% O2 (PFO blood flow only). Pulmonary artery pressure will also be measured using ultrasound. Subjects will undergo an intravenous blood draw. We will take 21 mL (~2 Tablespoons) of IV blood (~1.25 hrs)

Subjects will perform pulmonary function testing. (15min)

The subjects will be instrumented with forehead oxygen saturation monitor, ECG and blood pressure cuffs and baseline ultrasound measures of cardiac output, pulmonary pressure and left and right heart function measures will be made before breath hold (~30 min)

Subjects will ingest a placebo or sildenafil (50 mg oral) and wait until peak effect (~1hr), and then breathe low oxygen and high carbon dioxide gas mixtures that mimic blood gases during a breath hold with measure of cardiac output, pulmonary pressure and left and right heart function measures made every 2 minutes and during the last minute of the subjects breath hold (based on the average length the subject reports to be able to hold their breath) (~1.5 hrs).

Visit 2 Overview: **Subjects will ingest either a placebo or sildenafil (50mg, oral) (whatever they didn't take during visit 1) wait an hour for peak effect and then and then breathe low oxygen and high carbon dioxide gas mixtures that mimic blood gases during a breath hold, during which ultrasound measures of the heart and lungs will be made (~2 hrs)**

Visit 2 Details:
The subjects will be instrumented with forehead oxygen saturation monitor, ECG and blood pressure cuffs and baseline ultrasound measures of cardiac output, pulmonary pressure and left and right heart function measures will be made before breath hold (30 min).

Subjects will ingest a placebo or sildenafil (50 mg oral) and wait until peak effect (~1hr), and then breathe low oxygen and high carbon dioxide gas mixtures that mimic blood gases during a breath hold with measure of cardiac output, pulmonary pressure and left and right heart function measures made every 2 minutes while the subjects are breathing low oxygen and high carbon dioxide gas mixtures (~10 minutes) that mimic blood gases during a breath hold, (based on the average length the subject reports to be able to hold their breath) (~1.5 hrs).

Experienced SCUBA Diving Subjects (Objectives 4 & 5**)**

Visit 1 Overview: Subject consent, screening, lung function and anthropometrics. Subjects will perform a dive and then post dive they will ingest either a placebo or sildenafil (50mg, oral), wait an hour for peak effect and then ultrasound measures of the heart and lungs will be made (~5 hrs). Subjects will utilize SCUBA equipment owned and maintained by the Croatian Navy.

Visit 1 Details:
Subjects will undergo informed consent, fill out health history questionnaire (~45 min. Five to 10 hours prior to the dive start time, subjects will be given a telemetric pill to swallow prior to testing. The telemetric pill will be used to measure pre- and post-dive core temperatures in the divers. If the subjects do not swallow the core temperature pill, they will be ask to insert the pill as a suppository the morning of testing.

Subjects will be comprehensively screened for any cardiac abnormalities. Amount of blood flow travelling through the PFO and IPAVA will be graded using saline contrast echocardiography while the subject is breathing room air (PFO and IPAVA blood flow) and breathing 100% O2 (PFO blood flow only). Pulmonary artery pressure, cardiac output and right and left heart function will also be measured using ultrasound. Subjects will undergo an intravenous blood draw. We will take 21 mL (~2 Tablespoons) of IV blood (~1.25 hrs)

Subjects will perform pulmonary function tests. (15min)

Subjects will perform dives to depths up to 30 meters and lasting up to 75 minutes with decompression profiles determined using V-planner software according to the varying permeability model (~1.25 hrs). The Dive Master will ensure that all divers have a 'dive buddy', a SCUBA-certified individual of similar or greater experience than the subject. The subject will be encouraged to identify a 'dive buddy' to accompany them, but in the event that the subject is unable to secure a 'dive buddy', the Dive Master will ensure that the subject has a 'dive buddy' for the dive.

Subjects will ingest a placebo or sildenafil (50 mg oral) upon resurfacing and coming aboard dry land. During the hour wait until peak effect, the subjects will be instrumented with a forehead oxygen saturation monitor, ECG and blood pressure cuffs. Pulmonary artery pressure, cardiac output and right and left heart function will also be measured using ultrasound. In addition, a post-dive venous blood draw of 21 mL will be taken. This blood draw will occur 1 hour after placebo or sildenafil administration. (~1.5 hrs).

Visit 2 Overview: Subjects will perform a dive and then post dive they will ingest either a placebo or sildenafil (50mg, oral), wait an hour for peak effect and then ultrasound measures of the heart and lungs will be made (~3 hrs). Subjects will utilize SCUBA equipment owned and maintained by the Croatian Navy.

Visit 2 Details:
Five to 10 hours prior to the dive start time, subjects will be given a telemetric pill to swallow prior to testing. The telemetric pill will be used to measure pre- and post-dive core temperatures in the divers. If the subjects do not swallow the core temperature pill, they will be ask to insert the pill as a suppository the morning of testing. Prior to the dive, subjects will have 21 mL of venous blood drawn. After, subjects will perform dives to depths up to 30 meters and lasting up to 75 minutes with decompression profiles determined using V-planner software according to the varying permeability model (~1.25 hrs). The Dive Master will ensure that all divers have a 'dive buddy', a SCUBA-certified individual of similar or greater experience than the subject. The subject will be encouraged to identify a 'dive buddy' to accompany them, but in the event that the subject is unable to secure a 'dive buddy', the Dive Master will ensure that the subject has a 'dive buddy' for the dive. Subjects will utilize SCUBA equipment owned and maintained by the Croatian Navy.

Subjects will ingest a placebo or sildenafil (50 mg oral) upon resurfacing and coming aboard dry land. During the hour wait until peak effect, the subjects will be instrumented with forehead oxygen saturation monitor, ECG, and blood pressure cuffs. Pulmonary artery pressure, cardiac output and right and left heart function will also be measured using ultrasound. In addition, a post-dive venous blood draw of 21 mL will be taken. This blood draw will occur 1 hour after placebo or sildenafil administration. (~1.5 hrs).

Description of Data Collection Procedures

- **Comprehensive ultrasound screening** (Day 1 for Breath Hold and SCUBA divers):

An intravenous catheter will be placed in the subject used for blood draws and bubble injection. Subjects will sit in the left lateral decubitis position for ultrasound screening. An agitated saline contrast injection will be made while transthoracic saline contrast echocardiography (TTSCE, see below) is performed on the subject to evaluate extent of blood flow through IPAVA and PFO. This will be repeated while breathing 100% O2 for 10 minutes. Subject will be asked to perform a Valsalva maneuver while breathing room air and 100% O2. This maneuver enhances blood flow across the PFO. Multiple saline contrast injections (up to 3) may be performed to verify bubble grades/presence of a PFO. In addition, pulmonary artery pressure and stroke volume will be measured with ultrasound.

- **Pulmonary Function Tests** (Day 1 for Breath Hold and SCUBA divers):

Subjects will perform standard non-invasive spirometry to measure a maximal inspiratory and expiratory flow-volume loop, forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV1), and mid expiratory flow (FEF25-75%). These tests will require the subject to blow in and out of a mouthpiece connected to a computerized flowmeter (pneumotachometer). This is a routine clinical test performed in pulmonary function labs in the United States.

- **Transthoracic Saline Contrast Echocardiography (TTSCE)** (Day 1 & 2 for Breath Hold and SCUBA divers): Echocardiography requires a medical sonographer to place a small probe against the subject's ribcage, which transmits and receives sound waves to produce images that are captured and stored on a computer. Saline contrast is made by manually agitating (mixing) 3-5 ml of sterile saline and 1 ml of air to create a suspension (mixture of liquid and gas) of very small bubbles called microbubbles. This suspension is injected through an IV, which allows us to detect the transpulmonary passage of microbubbles.

- **Intravenous Catheter (IV) and blood draw** (Days 1 & 2 for Breath Hold and SCUBA divers):
We will place a 20-22 gauge (small diameter) IV into a vein in the subject's arm that will be used for the rapid injection of the agitated sterile saline for TTSCE. This catheter will also be used to measure serum inflammatory mediators. Blood will be centrifuged and de-identified serum stored at -80°C until assayed by ELISA kit. For the breath hold dive arms, blood will be drawn once on the first visit pre-breath hold (21 mL). For the SCUBA arm, blood will be drawn pre- and post-dive for both visits (21 mL per blood draw x4 blood draws). Total IV blood draw is therefore approximately 84 mL for SCUBA subjects.

•**Breathing low oxygen and high carbon dioxide** (hypercapnic hypoxia) to mimic breath hold (Days 1 & 2, breath hold and breath hold control subjects only):

Subjects will take either sildenafil or a placebo, and will be instrumented during the ~1 hour wait till peak effect. After being instrumented with ECG, forehead saturation and blood pressure monitoring equipment, subjects will breathe room air for 5-10 minutes to measure baseline breathing. During the first 5 minutes of the hypercapnic hypoxic conditions, subjects will breathe room air through a 2-way non-rebreathing Hans Rudolph mouthpiece. During the resting period end tidal (expired) Oxygen will be clamped at 100 mm Hg, while end tidal (expired) carbon dioxide will be maintained at resting levels (~30-40 mm Hg). Upon completion of the baseline measurements, end tidal carbon dioxide will be increased while end tidal oxygen will be reduced.

The values for oxygen and carbon dioxide for breath hold divers will be changed to 45-50 mm Hg CO2 and 50-60 mm Hg O2 and held at this level for approximately 5 minutes while ultrasound measures are made, then subsequently changed to 55-60 mm Hg CO2 and 30-40 mm Hg O2 and held at this level for approximately 5 minutes, while ultrasound measures are made. These values of oxygen and carbon dioxide were chosen based on previous values published in breath hold divers by my collaborating investigator Zeljko Dujic (Willie et al., J Cereb Blood Flow & Metab 2015).

The values for oxygen and carbon dioxide for breath hold diver controls will be changed to 45-50 mm Hg CO2 and 60-70 mm Hg O2 and held at this level for approximately 5 minutes while ultrasound measures are made, then subsequently changed to 55-60 mm Hg CO2 and 40-50 mm Hg O2 and held at this level for approximately 5 minutes, while ultrasound measures are made. The values used for controls are based off of previous work by our group at altitude (Elliott et al., J Appl Physiol 2015). End tidal oxygen and carbon dioxide levels will be maintained using an end-tidal forcing (ETF) system, (Airforce, Endtidal Forcing System), (Querido et al., 2013; Bain et al., 2013; Foster et al., 2014). (~30 minutes for breathing gases and 1 hour of wait time for drug plasma levels to peak).

• **SCUBA diving** (Days 1 & 2, SCUBA subjects only): Subjects will perform dives to depths up to 30 meters and lasting up to 75 minutes with decompression profiles determined using V-planner software according to the varying permeability model. Subjects will be highly experienced divers. The Dive Master will ensure that all divers have a 'dive buddy', a SCUBA-certified individual of similar or greater experience than the subject.

The subject will be encouraged to identify a 'dive buddy' to accompany them, but in the event that the subject is unable to secure a 'dive buddy', the Dive Master will ensure that the subject has a 'dive buddy' for the dive. A 'Dive Buddy' will be required to provide evidence of appropriate SCUBA certification and ensure that they have not completed a recent dive. Subjects will ingest a placebo or sildenafil (50 mg oral) upon resurfacing and coming aboard dry land. During the hour wait until peak effect, the subjects will be instrumented with forehead saturation, ECG and blood pressure cuffs (~1.5 hrs).

Core Temperature: Core temperature (Tcore) will be measured using a Vital Sense Monitor and a telemetric pill (JonahTM ingestible temperature sensor, Mini Mitter Inc, Bend OR). The sterile telemetric pill will be activated by study staff then will be taken orally (with liquid) the 5-10 hours prior to testing or the volunteer will self-insert the pill into the rectum the morning of testing.

D. Research Population & Recruitment Methods & Compensation

Sample size, Population and Recruitment Methods:

1. Sample Size and Data Analyses: Necessary sample size was determined with our primary outcome variables (Pulmonary artery systolic pressure, saline contrast bubble scores, TNFa levels) using our preliminary data, previously published work and *a priori* power analysis. Using G-Power and information about a physiologically-meaningful effect from published studies (i.e. differences in pulmonary artery systolic pressure by 25%, TNFa level differences between PFO+ and PFO- subjects of 50%, a desired power of 0.80, and alpha (0.05), we determined that n = 8-10 individuals of the same sex for each study group (Breath hold and SCUBA divers) would be sufficient to test our hypotheses, using a repeated measures design. Thus, we are asking to recruit up to 20 male subjects (10 with and 10 w/o PFO); and up to 20 female subjects (10 with and 10 w/o PFO) for the breath hold studies. We are also recruiting up to 20 male and up to 20 female age matched control subjects for the breath-hold studies as our control group., We are also requesting to recruit up to and an additional 25 male subjects (12-13 with and 12-13 w/o PFO); and up to 25 female subjects (12-13 with and 12-13 w/o PFO) for the SCUBA studies, for a grand total of 130 subjects, to account for subject attrition and variability in measures.

Table 1

Inclusion	Exclusion
Men and women aged 18-65 recruited from patients in the surrounding Spilt Croatia community who are elite breath hold divers and highly experienced SCUBA divers, and or age, sex and PFO-matched controls from the surrounding Split Croatia area and the surrounding Eugene/Springfield area for breath hold dive studies only.	Previous history of coronary artery disease (ischemic heart disease such as angina, heart attack, myocardial infarction). Currently taking medications or herbal supplements for any heart or respiratory disease that they cannot stop taking for 48hrs prior to testing (seasonal allergy medication not included in exclusion medications). Anyone who is pregnant or trying to become pregnant. Individuals taking Sildenafil or products similar to Sildenafil, such as Tadalafil (Cialis) and Vardenafil (Levitra/Staxyn). Individuals taking NITRATES and other Nitric Oxide donors. If subjects can refrain from using PDE-5 inhibitors for 48 hours before each study day then they will be allowed to participate. Previous history of any condition that would prevent the subject from breathing low oxygen and high carbon dioxide mixtures that mimic a breath hold. (**breath hold diving subjects only**). Previous history of any condition that would prevent the subject from performing a SCUBA dive. (**SCUBA diving subjects only**). Control breath-hold dive subjects can't have previous breath hold diving experience Subjects will be excluded from telemetric pill ONLY if they have a history of obstructive diseases of the gastrointestinal tract including diverticulosis, diverticulitis, inflammatory bowel disease, peptic ulcer disease, Crohn's disease, ulcerative colitis, or previous GI surgery.

2. Detailed Recruitment information:

Breath hold divers, controls and experienced SCUBA divers will be recruited using existing contact information by our collaborators in Split. Some control subjects for the breath-hold arm of the study (NOT SCUBA DIVING arm) will be recruited from the Eugene/Springfield area. Additionally, subjects will be provided information about the opportunity to participate in this research by word of mouth and announcements by coaches and affiliated diving personnel associated with this select group of individuals. Breath-hold control subjects will be informally recruited by word of mouth and announcements by coaches

as well, but they will be excluded if they have previous experience with breath-hold diving. In Eugene, breath-hold control subjects will be recruited by recontact from previous studies, word of mouth, and announcements by coaches, but they will be excluded if they have previous experience with breath-hold diving. Breath hold divers will be asked if they have anyone in mind that might be interested in breath-hold diving that have no previous experience doing it before, thus, helping us collect a control group.

While word of mouth and preliminary information about the study will be distributed by coaches and others in the diving community, formal recruitment will be conducted upon individuals contacting the research team. The research team will review the details of the experiment and the inclusion/exclusion criteria with each subject to allow them to make a decision regarding participation independent of peer influence. Our collaborators in Split are fluent in both Croatian and English, and will be able to explain all study procedures, data handling, and consent process in Croatian for those subjects who are not fluent in English. Control subjects from the Eugene/Springfield area will use English versions of the consent form.

3. Screening at each study visit: All subjects with child bearing potential will be required to take a urine pregnancy test before each visit begins. A positive test at any time will result in exclusion. Prior to the first visit each participant will be questioned regarding health history and current medications. Participants will be excluded if they meet any of the exclusion criteria listed in Table 1. For inclusion in core temperature measurements, subjects will be screened for the history of obstructive GI disorders during the consent meeting, during which they will be asked if they have an history of any of the GI disorders/diseases (including diverticulosis, diverticulitis, inflammatory bowerl disease, peptic ulcer disease, Crohn's disease, ulcerative colitis, previous GI surgery). This will be addressed when going through the exclusion criteria with subjects.

4. Compensation:

Subject Compensation (maximum of $50 for US subjects or 330 Croatian Khuna (HRK)) for either the breath hold diving and SCUBA studies in Croatia):

- **Visit #1 (Breath Hold, Breath Hold Control and SCUBA Subjects): $30.00 for US subjects or 220 Croatian Khuna for Croatian subjects (HRK))**: Completing the informed consent and health history questionnaire and echocardiographic screening and blood draw will pay $5.00. The pulmonary function tests will pay $5.00. Subjects who choose not to participate upon review of the informed consent or are excluded based on any findings during this visit will be paid for their degree of visit completion, that is $5.00 if the subject decides not to participate after reviewing the informed consent; $ 10.00 for participation in the screening procedures; total compensation for reviewing the informed consent and participating in screening is $10.00. Subjects will be paid $20 for either breathing low oxygen and high carbon dioxide or for performing the SCUBA dive. Compensation will be converted from USD to local currency upon arrival to Croatia. Estimates for payments are listed here based on current rates of exchange.
- **Visit #2 (Breath Hold, Breath Hold Control and SCUBA Subjects): $20.00 for US subjects or 110 Croatian Khuna (HRK))**: Subjects will be paid $20 for either breathing low oxygen and high carbon dioxide or for performing the SCUBA dive.

Any subject that withdraws (or is withdrawn) before the end of the study will receive prorated payment up to the point of completion.

E. Informed Consent Process

The Informed Consent document provided to subjects will be posted to the regulations.gov website in compliance with OHRP guidelines and 45 CFR 46.116(h).

Informed consent will be administered to each subject by the primary investigator and colleagues. For potential subjects who are only fluent in Croatian and not English, a native Croatian speaker from Dr. Dujic's lab will perform the informed consent process. The Informed Consent document provided to subjects will be posted to the regulations.gov website in compliance with OHRP guidelines and 45 CFR 46.116(h). English consent forms and English consent will be used for subjects in the US.

The primary investigator is well versed in the process of informed consent and has trained his co-investigators thoroughly on how to best perform this procedure. The PI will ensure that all investigators obtaining consent have experience in the informed consent procedure and are capable of adequately discussing the related physiology, study procedure and potential risks.

The researcher will first verbally explain the study in its entirety and in doing so walk through the informed consent in person. Subjects will then be given a sufficient length of time to read through the informed consent form privately and instructed/encouraged to write down or remember any questions/concerns they may have. Afterward, the researcher will rejoin the subject and address any question or concern they may have while subsequently going back through the informed consent form with the subject and obtaining any needed initials and signature at the end of the document. Furthermore, the investigator will verbally address any questions the subject may have regarding the seriousness and/or likelihood for the occurrence of the risks described in the Informed Consent Form. The research team will address probability and severity of any adverse reactions with the subject by carefully explaining the statements regarding probability and severity contained within the Informed Consent. The investigator will also provide appropriate statistics (where available) regarding the probability of adverse reactions. The investigator also advises the subject of what he/she can expect to feel during a particular procedure, for example, during contrast injection. Investigator further explains any questions regarding physiology or reason for a particular procedure in plain language.

On the day before ALL visits, the subject will be telephoned or e-mailed (depending on preference) to confirm participation and as a reminder to 1) not drink caffeine for 12 hours before each study day, 2) not exercise or drink alcohol for 24 hours before each study day and 3) not eat for 2 hours before arriving for each study day. Male subjects will be asked to go shirtless for all studies involving echocardiography. Female subjects will need to wear a sports bra for all studies involving echocardiography. To minimize any risk of embarrassment both male and female subjects will be allowed to wear a loose fitting shirt (provided by the researchers) that allows the upper body to be covered but also allows for imaging of the heart and the placement of small electrodes that record heart rate.

F. Provisions for Participant Privacy and Data Confidentiality

Each subject folder will be stored in a locking file cabinet inside the primary investigators locking office located at the Split Medical School until Dr. Lovering travels back to the United States. Subsequently, all hard copy data will be scanned into electronic format while in Croatia and saved on a password protected and fingerprint protected locked hard drive and hand carried by Dr. Lovering back to the United States. All hard copies will be left with collaborator Dr. Zeljko Dujic, who will store these hard copies indefinitely. All hard copies generated in the US will be kept in the Cardiopulmonary and Respiratory Physiology Lab in locked filing cabinets inside of a locked laboratory. These electronic format data will subsequently stored inside the primary investigators locking office located at the University of Oregon Cardiopulmonary and Respiratory Physiology Lab. The de-identified data will be kept for at least 7 years after publication, per NIH guidelines. In the unlikely event the data are not published, they will be kept for at least 10 years after collection. This will ensure sufficient time for publication after data have been collected considering some trainees take up to 6 years to graduate, and often publication does not occur until many years after graduation.

Each subject will be assigned an ID using a random number code system consisting of three to five letters describing the study (e.g., APNEA or SCUBA) and a random, non-repeating number (1-1000). This ID

will be associated with their unique folder, which will contain all study documents and data collected including all associated forms (i.e. informed consent document).
The primary investigator will maintain a subject ID key capable of identifying subject IDs to subject names and contact information to provide us with the ability to identify subjects as additional questions or research findings arise. This ID key will be kept in a locked filing cabinet also within the office of the primary investigator. No contact information will be stored with subject data.

De-identified data may potentially be shared with other investigators for research purposes.

G. Potential Research Risks or Discomforts to Participants

Confidentiality:

If data is lost or stolen, subjects could experience invasion of privacy. To minimize the potential invasion of privacy, we are not collecting social security numbers so that the potential economic impact is greatly minimized. All of our files will be kept in a locked filing cabinet to prevent theft and data will be de-identified. Data acquired on computers will be password protected. As such, the probability of the adverse outcomes discussed above is low, and the severity is minimal.

Psychological:

Male subjects will be asked to go shirtless for all echocardiography and exercise studies. Female subjects will need to wear a sports bra for all echocardiography and exercise studies. Female subjects will be allowed to wear a loose fitting scrub top (provided by the researchers) over their sports bra that allows for echocardiographic imaging and EKG electrode placement. Accordingly, both male and female subjects could potentially feel embarrassed or have modesty issues by being shirtless (males) or when only wearing a sports bra (females). To minimize the risk of embarrassment or modesty issues, both male and female subjects will be allowed to wear a loose fitting scrub top (provided by the researchers) that allows for concealment of upper body but also allows for echocardiographic imaging and EKG electrode placement. Female subjects will still be allowed to wear the sports bra with the scrub top which will allow for required instrumentation, but will also allow the subject to cover up as much as possible for the study. Both males and females may wear sweat pants or shorts, i.e. whatever makes the subject comfortable. As such, the ***probability*** of the adverse outcomes discussed above is low, and the ***severity*** is minimal.

Physiological:

Pulmonary Function Tests: Risks associated with pulmonary function testing include shortness of breath, cough, dizziness, and possible loss of consciousness. To minimize risks, the co-investigators will administer all pulmonary function tests and allow subjects to rest between measurements. Lung function testing performed in our lab is routinely and repeatedly done and performed in pulmonary function labs all over the world according to American Thoracic Society and European Respiratory Society standards. These tests can be stopped at anytime if cough, dizziness, shortness of breath, or possible loss of conscious occurs. Co-investigators will be monitoring the subjects for any signs of discomfort. The probability and severity of these risks is very low. These tests can be stopped at anytime if cough, dizziness, shortness of breath, or possible loss of consciousness occur. Co-investigators will be monitoring the subjects for any signs of discomfort.

Intravenous catheter: Risks associated with placement of an intravenous catheter (IV) include pain and/or bleeding during placement, vasovagal syncope, hematoma (pooling of blood under the skin), infection, and vessel blockage. The placement of the IV may cause some discomfort with rare bleeding or bruising at the puncture site. It also carries the risk of infection. To mitigate risks associated with vasovagal syncope, the subject will be safely positioned upright and sitting in an IV chair, and the subject will be continually monitored by Dr. Lovering and/or a member of the research team. Dr. Lovering or trained member of the research team will place IVs. We use aseptic techniques, sterile needles and IV catheters, so the probability

of experiencing any severe side effects such as infection is low and is not different from the risks of an IV being placed for the purpose of drawing blood for a routine clinical test. Some individuals may also feel like fainting at the site of needles of blood (vasovagal response). As such, the ***probability*** of the adverse reactions discussed above is low, and the ***severity*** is minimal.

Blood Removal: Risks associated with the removal of blood include an aversion to seeing blood that could result in nausea, vasovagal syncope, increased stress, and/or feeling faint. To mitigate these potential risks, the subject is continually monitored by Andrew Lovering PhD and colleagues during IV placement. In addition, the subject is safely and comfortably positioned on either a gurney or IV chair. In this way, the potential risk of vasovagal syncope (i.e., fainting) is mitigated. As such, the ***probability*** of the adverse reactions discussed above is low, and the ***severity*** is minimal.

Saline Contrast Echocardiography: The PI has been using saline contrast echocardiography since 2003 to detect blood flow through intracardiac and intrapulmonary shunts. Risks: transient dizziness associated with agitated sterile saline injection in patients with cardiac shunting. With respect to exercise, the Principal Investigator has >10 years of experience using TTSCE in a research setting. In 4 years (2003-2007) at the University of Wisconsin Madison, approximately 60 human (male and female) subjects (including 8 subjects with a patent foramen ovale) were tested without a single adverse event related to TTSCE. Additionally, research done at the University or Oregon between 2008 and 2019 has involved >200 subjects using TTSCE at rest and during exercise without incident related to the TTSCE. We will use agitated sterile saline without preservatives. Furthermore, we will use a minimal volume (3-5 mL) of sterile saline. Dr. Lovering or a trained member of the research team will perform saline contrast injections, while a trained and licensed ultrasonographer will perform echocardiography. The ultrasonographer will inspect the ultrasound machine for proper and safe functioning prior to use, and will identify any indications that the ultrasound machine may be functioning abnormally. In the event of such identification, repair and non-routine maintenance will be performed by a representative of the ultrasound manufacturer. Routine maintenance will be performed as needed by the ultrasound technician.

Mixed saline (saltwater), either alone or with 5% sugar in water **has been used to help see the ultrasound pictures (echocardiogram) for over thirty years**. Saline contrast bubble injections are routinely used to screen for the presence of a patent foramen ovale in the clinic. The American Society of Echocardiography Guidelines (2014) state that "…life threatening reactions are rare (<1 in 10,000)" when using contrast injections (including bubbles with protein shells) and The European Association of Echocardiography (2009) has stated that "… the evidence shows that contrast echocardiography is very safe in clinical practice." And this includes using stabilized bubbles with protein shells and we only use non-stabilized saline contrast bubbles in our lab. We only use a small amount of air mixed with saline, thus the ***probability*** of any ***severe*** adverse reaction is very low. Given the evidence presented above, the ***probability*** of the adverse reactions discussed above is low. Although the ***severity*** of arterial gas emboli is high, given the amount of air used and the short life span of intravascular bubbles of this size, the likelihood of the constellation of unfortunate events required for a serious adverse reaction to occur is very small.

Breathing low oxygen (hypoxia) and high carbon dioxide (hypercapnia): Risks associated with exposure to hypoxia or hypercapnia during rest include headache, nausea, fatigue, dizziness and shortness of breath and the remote possibility of central nervous system impairment, insomnia, pulmonary and cerebral edema, and death. Subjects will be monitored with an electrocardiogram and peripheral estimate of arterial oxygen saturation using a forehead monitor. Exposure to hypoxia at rest will be brief to minimize the impact of low oxygen. The principal investigator has extensive experience conducting exercise tests with subjects breathing hypoxic gas mixtures in a research setting (Amann, Eldridge et al., 2006; Romer, Havercamp et al., 2007; Lovering 2007; Lovering, Romer, et al., 2008; Lovering et al., 2011; Laurie et al., 2010; Elliott et al., 2015). Although the severity of risks breathing hypoxic and hypercapnic gas is potentially high, given the

short duration of exposure and careful monitoring by the research team, the likelihood of a serious adverse event to occur is very small.

Risks associated with core temperature pill: The risks of using the temperature pills include mechanical injury to the mucus membranes if adequate care is not used. Risks of the temperature pill will be minimized by explaining the procedures to the volunteers. Additionally, ample lubricant (suppository) or water (oral) will be provided to the volunteer. Volunteers with history of obstructive diseases of the gastrointestinal tract including diverticulosis, diverticulitis, inflammatory bowel disease, peptic ulcer disease, Crohn's disease, ulcerative colitis, or previous GI surgery will not use a telemetric pill. These risks only apply to subjects in the SCUBA arm.

Risks for subjects undergoing SCUBA diving: During the research, examinees will dive once to a max depth of 30 meters. Dive time will be a max of 75 minutes. At 30 meters (100 feet), the maximum bottom time before decompression limits are reached is 20 minutes (PADI dive table). When decompression limits are reached or exceeded, a decompression stop on the reascent is needed. Divers can safely exceed recreational decompression limits by adding a decompression stop, as decompression stops are designed to greatly minimize the risks of decompression sickness. Dive computers will be used so subjects can monitor how long they can stay at their current depth before requiring a decompression stop and determine length of a required decompression stop (standardized diving protocol determined by diving computer Galileo, Uwatec). The dive will be done in the presence of a safety diver, a paramedics doctor, while the doctors working at the Institute for hyperbaric medicine will be thoroughly informed about the diving protocol and schedule. Divers will utilize SCUBA equipment owned and maintained by the Croatian Navy. During the dive, the following adverse events are possible:

1. Decompression sickness – a condition in which air bubbles dissolved in tissues suddenly become released into bloodstream due to the decrease of the ambient pressure. Common symptoms include pain in the joints, extremities, dizziness, nausea, vomiting, extreme tiredness, paralysis, collapse and fainting. The incidence of decompression sickness among divers is generally low, ranging from 0.013% to 1.25%. Decompression sickness is primarily related to the depth and duration of the dive, as well as to the duration of decompression while resurfacing.
2. Arterial gas embolization – the bubbles can reach arterial system in people with defects in the interatrial wall of the heart.
3. Embolization – during the quick decompression, bubbles of oxygen can move from tissues into circulation.
4. Expansion lung injury and pneumomediastinum – can happen during the breath hold while resurfacing.
5. Oxygen narcosis – as a result of toxic effects of high oxygen pressure on nerve conducting, symptoms comparable to drunkenness may happen.
6. Oxygen toxicity – toxic effects of oxygen can be caused by the high quantity of absorbed oxygen, and can present as burning sensations in lungs, spasms, dizziness and vomiting.

Because we will be working with experienced divers utilizing equipment owned and maintained by the Croatian Navy, the probability of these risks is very low, although the severity is moderate.

Sildenafil (Viagra): Adverse reactions previously observed in individuals taking Sildenafil include moderate hypotension, light headedness, dizziness, priapism, sensitivity to light and potential visual disturbances. These reactions are extremely rare and largely associated with individuals who are using Sildenafil for the management of erectile dysfunction or pulmonary artery hypertension. We will further minimize these risks by using healthy subjects with no previous history of cardiovascular disease and use a standard clinical dose of 50 mg P.O. We will also exclude subjects from participation during Stage #1 if they report currently taking specific contraindicated medications (outlined in the exclusion criteria in Table 1). Dr. Zeljko Dujic, MD, Dr.

Lovering, and colleagues will be monitoring the subjects and ensuring the subject is not suffering from any negative side effects. Thus, the probability and severity of these risks is very low.

Safety Equipment available in the Laboratory of Dr. Zeljko Dujic in the department of integrative physiology at the University of Split School of Medicine in Split Croatia (where testing and screening will be performed) and the laboratory of Dr. Andrew Lovering in the department of human physiology at the University of Oregon: An AED, a spare AED battery and spare AED pads, bottled oxygen and face masks. A standard first aid kit is also available.

Emergency Procedures: According to American Heart guidelines, in the event of an adverse cardiopulmonary event, we will begin CPR, and call 211 in Croatia (which is equivalent to calling 911 in the US) and 911 for studies done in the US and will continue CPR as required until emergency medical personnel arrive.

Safety Monitoring: Subjects will be given clear instructions that they should notify the investigators immediately if they experience any of the above-mentioned risk symptoms. During all procedures, all subjects will be non-invasively and continuously monitored for vital signs using: 1) a 3 lead EKG to monitor electrical activity of the heart, and 2) a forehead probe to monitor arterial oxygen saturation and heart rate. During all other procedures subjects will be visually monitored for any signs of discomfort, distress or problems. Throughout all procedures, the investigators will continuously ask the subjects how they are feeling and how they are doing. During SCUBA dives, subjects will be accompanied by a 'dive buddy', a SCUBA-certified diver of similar or greater experience and able to assist the subject in the event of an emergency as is standard for the community.

H. Potential Benefits of the Research

This study will not improve the health of subjects and is only being done to gather information. This study will not improve the health of the general subject population. Completing the aims of this study will have the benefit of contributing to generalizable knowledge.

I. Investigator Experience

The PI and colleagues have been performing cardiopulmonary and respiratory physiology investigations at rest and during exercise in healthy and diseased populations at the University of Oregon for ~12 years. Dr. Dujic has been performing cardiopulmonary and diving physiology research for >25 years. Other investigators are all well qualified to work with human subjects having done so for many years in both laboratory and field settings. Dr. Lovering's CV is on file. Dr. Dujić is an internationally recognized expert on diving physiology and his CV is included.

The PIs train all graduate, undergraduate and technical personnel on all laboratory procedures and protocols. Ultrasound technicians, have worked with Dr. Lovering's and Dujic's groups for years and have performed thousands of resting and stress echoes in patients and research subjects.

Dr. Lovering & Dr. Dujić will work to ensure that all investigators are crossed trained on what is required for human subject research at each individual institution so that all procedures and activities are compliant with the rules and regulations for all institutions involved.

Dr. Dujić and his team are native Croatian speakers, as well as being fluent English speakers. This will allow for all study procedures, data handling procedures, and informed consent to be explained in either language depending on subject preference. Further, this will allow any important information, such as safety information, to be conveyed to subjects in the language they are most comfortable in. All Croatian collaborators are fluent in both English and Croatian and in some cases, Serbian and other languages. Thus, there will be no problem for our Croatian collaborators to completely understand each respective country's

ethical research practices and requirements. Because our Croatian collaborators will be fully informed of these practices and requirements, and because they all have PhDs, MDs and/or MD/PhDs, they will have absolutely no problem conveying the required information to any of the subjects.