The Examination of Inflammation, Iron Availability, and Patent Foramen Ovale as Factors that Influence Variability in Erythropoietin, Hemoglobin Mass, and Pulmonary Vascular Tone

by

Kaitlyn G. DiMarco

A dissertation accepted and approved in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in Human Physiology

Dissertation Committee:

Dr. Andrew Lovering, PhD, Chair

Dr. Christopher Minson, PhD, Core Member

Dr. Carsten Lundby, PhD, Core Member

Dr. Gabriella Lindberg, PhD, Institutional Representative

University of Oregon

Fall 2023

DISSERTATION ABSTRACT

Kaitlyn G. DiMarco

Doctor of Philosophy in Human Physiology

The Examination of Inflammation, Iron Availability, and Patent Foramen Ovale as Factors that Influence Variability in Erythropoietin, Hemoglobin Mass, and Pulmonary Vascular Tone

Individual variability in the cardiopulmonary system is often ignored in favor of focusing on group or treatment means. Erythropoietin (EPO) concentrations in response to renal hypoxemia, sea level hemoglobin (Hb) mass (Hb mass), and pulmonary vascular pressure changes in response to environmental stimuli are known to be markedly varied among individuals, yet very little research examines factors that may be responsible for that variability. Iron availability, immune system activity, and the presence or absence of a patent foramen ovale (PFO) are all factors that may play a modulatory role in EPO regulation, Hb mass, and pulmonary vascular tone regulation, yet our understanding of these factors in humans is largely unknown.

In Chapter IV, we demonstrate that carbon monoxide (CO) inhalation (COi) and hot water immersion (HWI) independently and in combination (COi + HWI) increased EPO concentration to the same degree. Importantly, the increase in EPO was driven by females. Baseline iron availability and inflammatory cytokine concentrations did not predict EPO concentration in response to COi or HWI. This study emphasizes the need for future studies to examine mechanisms underlying sex differences in EPO concentrations in response to COi and HWI.

In Chapter V, we show that some inflammatory cytokine concentrations and white blood cell counts moderately predicted Hb mass, but iron availability was the strongest predictor of Hb mass. The presence of a PFO did not alter Hb mass, although we do report lower ferritin in males with a PFO compared to males without a PFO. This study provides novel information that will provide direction to future research looking to utilize interventions aimed to alter Hb mass.

Lastly, in Chapter VI, we demonstrated that SCUBA dives not requiring decompression on the ascent do not increase pulmonary pressure or resistance. However, some cytokine concentrations increased post SCUBA diving, so future research should examine the role of inflammatory cytokines in modulating pulmonary pressure during SCUBA dives requiring decompression that elicit increases in pulmonary pressure and resistance.

This dissertation includes previously unpublished co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Kaitlyn G. DiMarco

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, OR
Colorado College, Colorado Springs, CO

DEGREES AWARDED:

Doctor of Philosophy, Human Physiology, 2023, University of Oregon
Bachelor of Arts, Molecular Biology, 2017, Colorado College

AREAS OF SPECIAL INTEREST:

High altitude physiology
Environmental physiology
Exercise & performance physiology
SCUBA diving physiology
Cardiopulmonary physiology
Immune & cardiopulmonary interactions

PROFESSIONAL EXPERIENCE:

Laboratory Instructor for Human Anatomy, Department of Human Physiology, University of Oregon, September 2019 – April 2022

Instructor of Record for Science of Sex, Department of Human Physiology, University of Oregon, June 2020 – July 2020

Instructor of Record for Medical Terminology, Department of Human Physiology, University of Oregon, June 2023 – August 2023

GRANTS, AWARDS, AND HONORS:

Peter O'Day Fellowship in Biological Sciences, 2022
Cum laude, Colorado College, 2017

PUBLICATIONS:

DiMarco KG, Beasley KM, Shah K, Speros JP, Elliott JE, Laurie SS, Duke JW, Goodman RD, Futral JE, Hawn JA, Roach RC, Lovering AT. No effect of patent foramen ovale on acute

mountain sickness and pulmonary pressure in normobaric hypoxia. Exp Physiol. 2022 Feb;107(2):122-132. doi: 10.1113/EP089948. Epub 2022 Jan 10. PMID: 34907608.

Lovering AT, Kelly TS, **DiMarco KG**, Bradbury KE, Charkoudian N. Implications of a patent foramen ovale for environmental physiology and pathophysiology: do we know the 'hole' story? J Physiol. 2022 Apr;600(7):1541-1553. doi: 10.1113/JP281108. Epub 2022 Feb 17. PMID: 35043424.

DiMarco KG, Chapman CL, Weiser NE, Matsell ER, Lucernoni KM, Chacon S, Grivette MMB, Halliwill JR, Lovering AT, Minson CT. Greater increase in erythropoietin in females in response to carbon monoxide inhalation and hot water immersion. *Manuscript in review with J Physiol*. 2023 Sept.

DiMarco KG, Betts AW, Bradbury KE, Matsell ER, Kelly T, Gideon EA, Duke JW, Lovering AT. Associations of hemoglobin mass with inflammation, iron status, and patent foramen ovale in females and males. *Manuscript submitted to AJP Heart & Circ.* 2023 Sept.

DiMarco KG, Futral JE, Lord RN, Barak O, Edward J, Drvis I, Glavičić I, Miloš I, Dujić Ž, Lovering AT. Non-decompression SCUBA diving increases circulating inflammation but not pulmonary pressure. *Manuscript in preparation*. 2023 Sept.

ACKNOWLEDGEMENTS

The work in this dissertation was supported by funding provided by the Wu Tsai Human Performance Alliance at University of Oregon (Chapter IV), United States Army Research Institute of Environmental Medicine (Chapter V), Research Logistics LLC (Chapter V), Peter O'Day Fellowship in Biological Sciences (Chapter V), PADI Foundation Diving Physiology Grant (Chapter VI), Fulbright Scholars Program (Chapter VI), University of Oregon College of Arts & Sciences International Research Travel Grant (Chapter VI), and Global Oregon Faculty Research Travel Program (Chapter VI).

In addition, I would like to extend my gratitude towards my dissertation committee for their continued guidance; past and present members of the Cardiopulmonary & Respiratory Physiology lab for their help with the research included in this dissertation including Karleigh Bradbury, Aaron Betts, Emma Matsell, Natasha Weiser, Margaret Grivette, Elizabeth Castillo, Hannah Feldmeier, Tyler Kelly, Prakrunya Subhasree Badrinarayan, Karina Shah, Mohini Bryant-Ekstrand, Kissy Sendrijas, Ben Whitson, Ryan Rojas, Garrett Clark, Kinsey Lovich, Aubrey Iniguez, Ben Bratland, Amanda Henny, Philip Tracy, Makayla Perez, Tucker Orman, Georgia Wolfe, Carolyn Hayes, Jordan Li, & Venice Loar; all of the ultrasonographers who helped with the ultrasound measures in this dissertation including Dr. Christopher Chapman, Joel Eben Futral RDCS, Doug Elton RDCS, Freddy Garcia RDCS, and Dr. Rachel Lord; Drs. Carrie McCurdy, Hans Dryer, Robert Guldberg, and Kelly Leguineche for allowing me the use of their lab equipment; Karen Needham and Lindy Comrada for help with organizing studies, wet lab training, and supply ordering; and Drs. JJ Duke and Igor Gladstone for their continued mentorship.

DEDICATION

To put it simply, this journey has been nothing short of challenging. I began my work in a human subject research lab in the Fall of 2019, where we were plunged into a global pandemic mere months later. Navigating the challenges of learning new research techniques and conducting my own studies in the context of a global pandemic was not a one-person job, and I would never have gotten to where I am without the incredible support of mentors, advisors, peers, and friends throughout these last four and a half years. I would like to start by extending my most sincere thank you to my advisor and mentor, Dr. Andrew Lovering. He took a chance on me as a graduate student and has provided me with countless opportunities to grow as a researcher, writer, communicator, traveler, and overall professional. I would also like to thank my incredible committee members for their continued guidance. Dr. Christopher Minson has been a second advisor over the years, and I can always count on his extreme enthusiasm about new research ideas. Dr. Carsten Lundby has been instrumental in honing a research technique integral to this dissertation, and Dr. Gabriella Lindberg's feedback and support on all things immune system has made me a stronger writer and scientist.

I owe particular thanks to my peers and undergraduate mentees, who have become incredible friends over the years. Karina Shah, Emma Matsell, and Natasha Weiser, I loved watching you all become incredible scientists and writers, and I thank you for allowing me to learn how to be a better mentor. To the other graduate students in my lab, thank you for providing so many laughs through all the challenges and being so willing to always provide help. Karleigh Bradbury and Aaron Betts, you have truly been incredible friends in and out of the lab. To my fellow graduate students not in my lab, especially Neeka Barooni, Emma Reed,

Mackenzie Kehmeier, Jessica Atencio, and Katie Lucernoni, your support and friendship both on campus and on the ski hill has been crucial to me making a home here.

Lastly, this would not have been possible by the support of my family and partner, Tom Boivin. Nothing brings me more joy and occasional laughter than listening to you all try to explain my research to other people. Without your support, I would have given up on this dream long ago. Tom, you were there to support me every single day of this journey and more than anyone listened to the tears, the triumphs, and everything in between. From the bottom of my heart, thank you, because this would not have been possible without your support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

This dissertation contains previously unpublished co-authored material. Chapters I, II, III, and VII, and VIII do not contain co-authored material and are written entirely by me with editorial assistance solely from my dissertation committee. Chapters IV, V, and VI contain co-authored material. Co-authors are listed at the beginning of each applicable chapter and include Karleigh E. Bradbury, Aaron W. Betts, Tyler Kelly, Emma R. Matsell, Natasha E. Weiser, Margaret M.B. Grivette, Dr. Christopher L. Chapman, Kathryn M. Lucernoni, Samantha Chacon, Dr. John R. Halliwill, Dr. Christopher T. Minson, Elizabeth A. Gideon, Dr. John W. Duke, Joel E. Futral, Dr. Rachel N. Lord, Dr. Otto Barak, Dr. Justin Edward, Dr. Ivan Drvis, Dr. Igor Glavičić, Ivana Miloš, Dr. Željko Dujić, and Dr. Andrew T. Lovering.

INTRODUCTION

The primary function of the cardiopulmonary system is to facilitate transport of gases, nutrients, and cells through movement of blood. The pulmonary arterial circulation delivers low oxygenated blood to the lungs. At the lungs, oxygen diffuses into the blood and binds to Hb in red blood cells and dissolves in plasma, while carbon dioxide diffuses out of the blood to be exhaled. Once blood returns to the left side of the heart, the systemic circulation delivers oxygenated blood to organs and tissues.

Blood is composed of plasma, red blood cells, white blood cells, and platelets. Plasma contains various proteins and ions and makes up approximately 54-59% of blood. Red blood cells are crucial for oxygen transport and make up approximately 40-45% of blood at sea level, and white blood cells regulate the immune response and make up approximately 1% of blood. Platelets are involved in the clotting process and make up a minute amount of the total blood volume.

Red blood cells are denucleated cells that functionally consist of Hb and cytoplasm. Hb is a protein that is composed of four subunits surrounding an iron molecule and is required for oxygen transport in the blood, but Hb can also bind other gases like CO, which will outcompete oxygen for Hb binding. Red blood cells are regulated by the protein EPO such that EPO is maintained at low concentrations at sea level to maintain red blood cell mass, also called Hb mass, in the face of red blood cell turnover. EPO is produced mainly by the kidney in adult humans, and when oxygen delivery to the kidney is compromised, significant increases in circulating EPO occur to increase Hb mass and restore oxygen delivery. Most research on EPO has examined EPO concentrations in response to normobaric or hypobaric hypoxia (Abbrecht & Littell, 1972; Milledge & Cotes, 1985; Berglund *et al.*, 2002; Ge *et al.*, 2002; Wojan *et al.*, 2021), but pertinent to this dissertation, there is growing interest in the use of COi and whole body heat stress as ways to stimulate EPO, increase Hb mass, and thereby increase aerobic exercise performance (Lorenzo *et al.*, 2010; Ryan *et al.*, 2016; Wang *et al.*, 2019*a*; Oberholzer *et al.*, 2019; Schmidt *et al.*, 2020; Rønnestad *et al.*, 2021, 2022).

The pulmonary and systemic circulatory systems are differently regulated. Regulation of the pulmonary circulation is largely passive and involves minimal neural control, unlike its systemic circulation counterpart. However, there are situations in which pulmonary vascular tone is actively regulated, most notably during hypoxic pulmonary vasoconstriction (HPV), which occurs when there are reductions in pulmonary arterial and alveolar oxygen tension. When alveolar hypoxia occurs globally, such as would occur at high altitude, increases in pulmonary vascular resistance and pressure occur due to the global vasoconstriction of the pulmonary arterioles. Importantly for this dissertation, SCUBA diving also results in an increased pulmonary pressure post-dive (Dujić *et al.*, 2006; Marabotti *et al.*, 2013), although the

mechanisms causing this increased pulmonary arterial pressure are unclear given that SCUBA diving while breathing air results in alveolar and arterial hyperoxia and not hypoxia.

A key aspect of the regulation of EPO concentration, Hb mass, and pulmonary vascular tone is the large degree of individual variability. The current understanding is that changes in circulating EPO concentration, sea level Hb mass, and pulmonary pressure changes are solely dependent on the stimulus; however, at a given stimulus there is still a notable degree of variability in these responses and baseline values in humans (Ge *et al.*, 2002; Swenson, 2013; Falz *et al.*, 2019; Oberholzer *et al.*, 2019; Rønnestad *et al.*, 2021, 2022; Skattebo & Hallén, 2022). The goal of this dissertation was to examine the potential roles of various immune system components, iron status parameters, and a PFO on these cardiopulmonary variables in females and males to determine if these factors modulate the cardiopulmonary system and help to explain inter-individual variability.

Despite comprising only 1% of total blood volume, white blood cells swiftly and effectively mount a strong response to foreign pathogens through the release of proteins called cytokines, which regulate immune cell recruitment, proliferation, and differentiation as well as other immune responses such as the fever response and inflammation. Inflammatory cytokines can negatively regulate EPO concentration in response to hypoxia (Fandrey & Jelkmann, 1991; Faquin *et al.*, 1992; La Ferla *et al.*, 2002), but no studies to date have examined this response in healthy humans or if cytokines negatively regulate EPO in response to COi or acute heat stress. Cytokines and white blood cells can also negatively regulate red blood cells independent of their effect on EPO (Fraenkel, 2017; Weiss *et al.*, 2019; Ganz, 2019), but similar to EPO, this relationship has not been well studied in healthy humans. Lastly, inflammatory cytokines have been shown to augment pulmonary vasoconstriction (Tsai *et al.*, 2004). Importantly, SCUBA

diving is a non-pathogenic situation in which inflammatory cytokine concentrations increase (Žarak *et al.*, 2021; Rocco *et al.*, 2021), but no studies to date have examined whether there is a modulatory role of inflammatory cytokines in pulmonary vascular tone regulation post-SCUBA diving.

Iron status, which we define as a combination of total blood iron, ferritin, and transferrin concentrations, is an important factor in regulating red blood cell production and development. Since Hb is a ferric protein, iron is crucial to the formation of red blood cells. Additionally, low intracellular iron has been shown to suppress HIF-2α, the primary transcription factor required for the increase in EPO concentration (Haase, 2013). Therefore, understanding iron status may help explain variability in EPO and Hb mass regulation.

Lastly, a foramen ovale is a right-to-left shunt present in all fetuses that functionally closes upon birth. When the infant begins to breathe, alveolar oxygen increases and the pulmonary vascular resistance subsequently decreases such that the pressure in the left atrium exceeds that in the right atrium, favoring functional closure of the foramen ovale. The foramen will anatomically close over time in the majority of infants but remains open in approximately 25-40% of the population (Hagen *et al.*, 1984; Marriott *et al.*, 2013; Elliott *et al.*, 2013), which is termed a PFO. Despite the PFO being a source of shunt, the presence of a PFO alone does not cause a significant degree of arterial hypoxemia (Lovering *et al.*, 2011; Duke *et al.*, 2020) that could result in a compensatory erythropoietic response or HPV. However, it is possible that the presence of a PFO alters expression of inflammatory cytokines, white blood cells, or iron status parameters. Data suggests a link between the presence of a PFO, iron deficiency, and increased stroke risk (Shovlin, 2014; Topiwala *et al.*, 2021), although this has not been confirmed, and no

data exists on whether the presence of a PFO alters the expression of various immune system components.

Accordingly, the overarching purpose of this dissertation was to examine factors that modulate EPO regulation, sea level Hb mass, and pulmonary vascular tone regulation, and in doing so, explain the individual variability in these responses (EPO and pulmonary pressure) and baseline values (Hb mass). The first objective addressed in Chapter IV was to examine EPO concentrations in response to COi, HWI, and COi + HWI in females and males. In addition, we wanted to determine what additional factors may explain the variability in EPO concentrations, including baseline inflammatory cytokine concentrations and baseline iron status. The second objective, addressed in Chapter V, was to quantify the associations of immune system components (white blood cell counts and cytokine concentrations), iron status parameters, and the presence or absence of a PFO on Hb mass in females and males. Lastly, the third objective, addressed in Chapter VI, was to determine whether two different depth and time SCUBA dive profiles increased pulmonary pressure and resistance. In addition, we wanted to quantify inflammatory cytokine concentrations pre- and post-SCUBA diving and determine the role of cytokines, nitric oxide (NO), and the presence of a PFO in modulating pulmonary pressure post-SCUBA diving.

HISTORICAL PERSPECTIVE

Cardiopulmonary physiology has a colorful history characterized by failure to accept new ideas. Many of these inaccuracies persisted for centuries, and much of our current understanding of cardiopulmonary physiology has only been elucidated in the last 150 years. These recounted stories are taken from various works of Dr. John West (West, 2011, 2015; West *et al.*, 2013) and Dr. Andrew Lumb (Lumb, 2005).

These pertinent histories begin as far back as ancient Greece with Claudius Galen, a Gladiator physician in the second century AD. He was the first documented scientist to describe the existence of the PFO. However, one of his most prominent ideas is also his most problematic. He believed that blood was made in the liver and from there traveled to the right side of the heart. He incorrectly believed that only some of the blood then traveled to the lungs, while most of the blood flowed through pores in the interventricular septum. The blood in the lungs was mixed with "pneuma" from the trachea and converted into a "vital spirit" in the heart, where it mixed with blood that traveled through the interventricular pores. "Galen's pores" as they would come to be called, persisted as the accepted reality of the cardiopulmonary system for nearly 1400 years, despite no scientist being able to find them.

In the 13th century AD, an Islamic scientist named Ibn Al Nafis directly refuted Galen's ideas. He found that there were no pores in the interventricular septum and therefore all blood flows through the lungs where it mixes with air. However, his ideas were largely ignored, allowing Galen's ideas to persist for a few hundred more years. Michael Servetus, a western physiologist who was apparently unaware of Ibn Al Nafis' work, supported Al Nafis' ideas that there are no interventricular pores in the heart. In addition, Servetus is credited with discovering that blood can change color when it passes through the heart, although he was unaware that oxygen was responsible for this color change. Unfortunately, Servetus was deemed a heretic, and he and his books were burned at the stake. Finally, in 1616, William Harvey published his findings that blood circulates continuously throughout the body and lungs. He is also credited with discovering the "lesser" and "greater" circulations, which we know as the pulmonary and systemic circulations, respectively.

The next sequence of stories recounts those individuals who discovered that oxygen was actually the "vital spirit" or "pneuma" that was crucial to life. In a set of rather gruesome experiments, in the 17th century, Robert Boyle, Richard Lower, John Mayow, and Robert Hooke experimented with vacuums and discovered that flames were extinguished, and animals could not survive. Lower also discovered that the color change in blood due to gases occurs in the lungs and not the heart. Then, in the 18th century, the theory of phlogiston emerged (phlogiston is defined as the part of air that combusts). During this time, Joseph black is credited with the discovery of carbon dioxide. Later, Joseph Priestly performed a set of experiments in which he burned mercuric oxide and noticed that mice thrived breathing this gas, further supporting the idea that there is a component of air that is vital to life. Carl Scheele supported the ideas of Priestly and discovered what he called "fire air". Finally, Lavoisier, a French scientist, discovered the actual chemical element of oxygen in 1777. He also correctly described the process of oxygen entering the lungs, carbon dioxide being expired by the lungs, and nitrogen as a passive medium that enters and exits the lungs without being consumed.

After John Dalton published his law of partial pressures in the 19th century (Dalton, 1826), Gustav Magnus discovered that oxygen and carbon dioxide could be dissolved in blood. Importantly, Lothar Meyer then discovered that oxygen binds to Hb in the blood and is not just transported by being dissolved. Later, in the mid-1800s, Hüfner found that Hb has a binding affinity of 1.34 mL oxygen per gram of Hb. Hüfner is the first to describe the association between oxygen tension and Hb saturation, but his oxyhemoglobin curve was not the sigmoidal relationship we know today.

At this point in history, scientists had discovered and/or correctly described the cardiopulmonary anatomy and the gases that are important in respiration, as well as how those

gases are transported in the blood. Next are the stories of those that studied the process of gas exchange at the lung. In the late 1800s, Christian Bohr published his oxygen secretion theory. His idea was that the lung actively secreted oxygen into the blood since his experiments showed that the oxygen tension of blood was higher than in the air. However, despite this incorrect theory of oxygen secretion, Bohr is credited with discovering the sigmoidal oxyhemoglobin dissociation curve. Haldane, a prominent respiratory and altitude physiologist at the time, was a staunch supporter of Bohr's ideas. However, one of Bohr's pupils, August Krogh, and Krogh's wife, Marie, refuted Bohr's oxygen secretion ideas and showed that oxygen passively diffuses from the lungs into the blood. August discovered that arterial oxygen tension was less than that of air, while his wife Marie investigated the diffusing capacity of the lung and created a technique we still use today – using CO to measure lung diffusing capacity. Marie and August Krogh conducted their work in the early 20th century, so it is surprisingly recent that we came to discover one of the most fundamental aspects of respiratory physiology.

In the late 19th century, Viault discovered that reduced arterial oxygen content (CaO_2) would stimulate erythropoiesis. Then, in 1911, Haldane led an altitude research expedition to Pikes Peak at 4300m. Despite his prevailing oxygen secretion ideas, he is credited with showing polycythemia with extended altitude exposure. It was not until decades later though that the mechanisms of this response and many of the other hypoxic responses would be discovered. In 1991, Dr. Gregg L. Semenza discovered hypoxia inducible factors, and he would go on to win the Nobel Prize in Physiology or Medicine in 2019 for his work in this area along with Peter J. Ratcliffe and William G. Kaelin. Importantly, his discovery of HIF occurred while studying the EPO gene (Semenza *et al.*, 1991). Human EPO was first isolated only a few years prior in 1977 (Miyake *et al.*, 1977; Kalantar-Zadeh, 2017).

Also in the 20th century was the discovery of hypoxic pulmonary vasoconstriction. Von Euler and Liljestrand demonstrated an increase in pulmonary pressure in cats ventilated with low oxygen in 1946 (Euler & Liljestrand, 1946). The first demonstration of HPV in humans occurred the year after by Motley (Motley *et al.*, 1947).

Overall, the stories above point to a long history of prevailing inaccuracies from leaders in the field, even amongst those with successful discoveries. Given the relatively recent discovery of much of what we know to be true in cardiopulmonary physiology, it is perhaps unsurprising that we have yet to fully examine modulators of these responses and individual variability. This history was provided to show that there are still many gaps in our understanding of cardiopulmonary physiology, particularly when working with intact humans.

BACKGROUND AND SIGNIFICANCE

Red blood cell and EPO regulation

Red blood cell production is largely regulated by the protein EPO. Baseline EPO production is important for the maintenance of Hb mass in the face of normal red blood cell turnover, and increased EPO production is crucial to the formation of new red blood cells (Jelkmann, 2011). EPO production is increased when oxygen delivery to the kidney is compromised. Oxygen sensitive renal cells, called renal fibroblasts, sense reduced oxygen delivery and respond by increasing EPO protein concentration (Jelkmann, 2011). The kidney is the primary site of EPO production, although the liver can also produce EPO (Haase, 2013). Oxygen delivery to the kidney can be reduced in numerous ways that target either renal perfusion and/or CaO_2. More recently in the world of performance physiology, there is increased interest in using CO and whole body heat stress to stimulate EPO, increase Hb mass, and

increase aerobic exercise performance (Lorenzo *et al.*, 2010; Ryan *et al.*, 2016; Wang *et al.*, 2019*a*; Oberholzer *et al.*, 2019; Schmidt *et al.*, 2020; Rønnestad *et al.*, 2021, 2022).

EPO is directly downstream of hypoxia inducible factor (HIF) pathways. HIFs are composed of an alpha and beta subunit, both of which are constitutively expressed. However, in the presence of oxygen, prolyl hydroxylases tag the alpha subunit for proteasomal degradation and render the HIF inactive. With hypoxia, prolyl hydroxylases are inhibited, allowing the HIF alpha and beta subunits to dimerize and act as transcription factors for numerous downstream targets. There are three alpha subunits of HIF currently known – 1, 2, and 3. EPO is primarily downstream of HIF-2α, which is also known as endothelial per-arnt-sim domain-containing protein 1 (EPAS1) (Jelkmann, 2011; Haase, 2013).

EPO is involved primarily in promoting the survival and differentiation of the erythroid precursors called colony forming units-erythroid (CFU-E) and blast forming units-erythroid (BFU-E) (Jelkmann, 2011; Nandakumar *et al.*, 2016; Bhoopalan *et al.*, 2020). These erythroid precursors have many surface receptors for EPO, while downstream erythroid precursors express fewer and fewer EPO receptors (Broudy *et al.*, 1991). When EPO binds its receptor, it initiates a signaling cascade by acting through numerous pathways but acts primarily through Janus Kinase 2/Signal transducer and activator of transcription 5 (JAK2/STAT5) signaling (Bhoopalan *et al.*, 2020). The downstream targets of these signaling cascades are numerous and largely involved in promoting cell survival by preventing apoptosis as well as providing negative feedback on EPO signaling (Bhoopalan *et al.*, 2020).

When there is a sustained reduction in renal oxygen delivery, Hb mass will increase through the actions of EPO. A notable example of this is during chronic sustained altitude exposure. EPO peaks in approximately 48 hours after the onset of hypoxia (Abbrecht & Littell,

1972; Milledge & Cotes, 1985; Garvican *et al.*, 2012), while it takes red blood cells approximately 1-2 weeks to fully mature and increase Hb mass (Garvican *et al.*, 2012; Ryan *et al.*, 2014). This is likely because EPO is involved upstream in the erythropoiesis process, and the maturation from CFU-E and BFU-E precursors to reticulocytes to functionally mature red blood cells takes days to weeks.

While EPO has been studied in both acute and chronic hypobaric and normobaric hypoxia (Abbrecht & Littell, 1972; Milledge & Cotes, 1985; Berglund *et al.*, 2002; Ge *et al.*, 2002), minimal studies have examined the response of EPO to CO (Wang *et al.*, 2019*a*; Schmidt *et al.*, 2020) or acute heat stress (Akerman *et al.*, 2017; Oberholzer *et al.*, 2019). Most studies examining hematological responses to heat stress or COi examine chronic changes in Hb mass (Ryan *et al.*, 2016; Wang *et al.*, 2019*a*; Oberholzer *et al.*, 2019; Schmidt *et al.*, 2020; Rønnestad *et al.*, 2021, 2022; Lundby *et al.*, 2023). Therefore, there more research needs to be done to examine acute mechanisms by which these interventions increase Hb mass. In addition, heat stress and COi presumably reduce oxygen delivery to the kidney via two different mechanisms, reduced renal perfusion and reduced CaO_2, respectively, yet no studies have examined whether heat stress and COi provide an additive stimulus for EPO production. Lastly, no studies have examined EPO concentrations in both females and males in response to interventions aimed to increase EPO concentration, so any sex differences in this response are entirely unknown.

Pulmonary pressure regulation

Pulmonary circulation is notably different from systemic circulation. Despite receiving the same cardiac output (Q), the regulation of the pulmonary circulation allows blood pressure to be 1/5 of that experienced by systemic circulation. Unlike its systemic counterpart, the pulmonary circulation is characterized by much less neural control, and the blood vessels have

much less muscle tone. Regulation of pulmonary circulation is therefore a largely passive process with blood flow distribution in the lung driven by the architecture of the pulmonary vasculature. To maintain pulmonary vascular pressures in the face of increased Q and therefore pulmonary blood flow, vessels can passively distend, and more vessels can be recruited to receive blood flow.

Pulmonary vascular tone can be actively regulated in some contexts, however. The most well studied example of this is HPV. As the name implies, during regional lung hypoxia the pulmonary arterioles constrict to redirect blood flow to better ventilated areas of the lung and thus maintain adequate ventilation/perfusion matching and optimal pulmonary gas exchange efficiency. However, when the alveolar hypoxia is global, as would occur at altitude, the global vasoconstriction results in an increased pulmonary vascular resistance and therefore an increased pulmonary arterial pressure (Swenson, 2013). HPV is primarily sensitive to decreases in alveolar oxygen tension but is also sensitive to decreased pulmonary arterial oxygen tension. Venous blood with low oxygen returning to the right side of the heart gets pumped to the lungs, and that blood can provide an additional, albeit less influential, stimulus for HPV in addition to alveolar hypoxia.

Interestingly, pulmonary pressure is reportedly elevated following SCUBA diving breathing air that requires decompression during the ascent (termed decompression SCUBA dives) (Dujić *et al.*, 2006; Marabotti *et al.*, 2013). SCUBA diving while breathing air results in hyperoxic lung and blood conditions due to the hyperbaria, which may not be initially expected to cause a vasoconstriction. Despite this, there is evidence that hyperoxia can redistribute pulmonary blood flow, which suggests that hyperoxia may cause an active redistribution of blood flow via a vasoconstriction mechanism (Melsom *et al.*, 1999). However, the increase in

pulmonary pressure is presumably due to some degree of sustained vasoconstriction independent of hyperoxia, since the response lasts hours after a dive (Marabotti *et al.*, 2013) when any effects of hyperoxia and other conditions experienced during a dive would be expected to be negligible. Importantly, this has not been confirmed, and whether the mechanisms responsible for the increase in pulmonary pressure with SCUBA diving mirror those that cause HPV is unknown. Lastly, it is unknown whether non-decompression SCUBA diving increases pulmonary pressure.

Roles of the immune system, PFO, and iron status on inter-individual variability

As the central focus of this dissertation identifies, the cardiopulmonary variables described above have a large degree of inter-individual variability. Increases in EPO concentration with hypoxia have been shown to vary from -41 to 400% (Ge *et al.*, 2002). While some of this response is stimulus driven (i.e., EPO increases to a greater degree at higher altitudes (Ge *et al.*, 2002)), at a given stimulus there is a large degree of inter-individual variability. Similarly, there is also a wide range of 'normal' physiologic values of Hb mass (Falz *et al.*, 2019; Goodrich *et al.*, 2020; Skattebo & Hallén, 2022). Even within one sex assigned at birth and when normalized to either whole body mass or lean body mass, Hb mass is markedly varied (Falz *et al.*, 2019; Goodrich *et al.*, 2020). Lastly, for a given hypoxic stimulus, there is a notable degree of variability in HPV (Swenson, 2013). While this dissertation does not directly study HPV, the similarities in the outward appearance of HPV and increases in pulmonary pressure post SCUBA diving led us to believe that there would be notable variability in the SCUBA response as well.

One potential source of the variability is immune system activity. Data suggest that inflammatory cytokines can regulate or modulate pulmonary pressure in response to hypoxia (Tsai *et al.*, 2004), red blood cells (Fraenkel, 2017; Weiss *et al.*, 2019; Ganz, 2019), and/or EPO

concentrations in response to hypoxia (Fandrey & Jelkmann, 1991; Faquin *et al.*, 1992). Additionally, iron is crucial for Hb formation and therefore plays a role in modulating Hb mass as well as regulating EPO concentration (Haase, 2013). Low iron availability can inhibit increases in EPO to preserve iron stores from being used to make more red blood cells (Haase, 2013). Therefore, iron status may play a crucial modulatory role in both Hb mass and EPO regulation.

Lastly, despite those with a PFO having the potential to have worse pulmonary gas exchange efficiency at rest (Lovering *et al.*, 2011; Duke *et al.*, 2020), under sea level resting conditions there is no evidence of arterial hypoxemia in those with a PFO nor any PFO driven differences in pulmonary pressure with normobaric hypoxia (Lovering *et al.*, 2011; Duke *et al.*, 2020; DiMarco *et al.*, 2021). Therefore, the presence of a PFO alone is not expected to alter Hb mass or pulmonary pressure regulation post-SCUBA diving due to hypoxemia, but the presence of a PFO may influence iron availability (Shovlin, 2014; Topiwala *et al.*, 2021) and/or expression of various immune system components and therefore indirectly alter Hb mass and/or pulmonary pressure post-SCUBA diving, although this remains unstudied.

STATEMENT OF PROBLEM

Historically, in the field of human physiology, individual variability to various physiological challenges is not well understood, with the majority of studies aiming to show significant differences in group or treatment means. Although cardiopulmonary responses are largely driven by their various stimuli, there are likely multiple reasons for the individual variability that are not yet appreciated or fully understood. For example, despite a good understanding of how altitude stimulates EPO via the HIF pathway, the mechanisms underlying the significant variability in EPO concentrations remain poorly understood. In addition, the study

of alternate forms of renal hypoxemia on EPO production is still in its infancy, and no studies that have examined EPO concentration in response to COi or heat stress have included females. Similar to EPO, there is a large degree of variability in Hb mass between individuals. While normalizing Hb mass to lean body mass can eliminate some variability (Falz *et al.*, 2019; Goodrich *et al.*, 2020), at a given lean body mass there is still an approximate range up to 300 grams of Hb within the same sex assigned at birth, which can represent roughly 25-60% of an individual's total Hb mass (Goodrich *et al.*, 2020). Lastly, mechanisms behind the post-SCUBA diving increase in pulmonary pressure are incompletely understood, and the mechanisms explaining the variability in pulmonary vascular tone regulation post-SCUBA diving are unknown. A more wholistic approach to understanding how all the integrated systems interact may better explain the reasons for the substantial variability in EPO production with renal hypoxemia, red blood cell regulation and Hb mass at sea level, and pulmonary pressure changes post-SCUBA diving.

PURPOSE AND HYPOTHESES

The primary purpose of this dissertation was to quantify the associations of various immune system components, iron status parameters, and the presence of a PFO with 1) circulating EPO concentration in response to COi and acute heat stress, 2) Hb mass at sea level, and 3) pulmonary pressure regulation post-SCUBA diving. We sought to determine the strength of these associations in an effort to better understand the individual variability in these values and responses to various environmental stimuli. Each aim had a primary cardiopulmonary outcome as well as an additional secondary outcome to reconcile the inter-individual variability in the cardiopulmonary outcomes.

Aim 1. Quantify serum EPO concentration in response to COi, HWI, and COi + HWI and determine if there are sex differences in this response. Quantify the association of these changes in EPO concentration with baseline cytokine concentrations and iron status.

Despite decades of EPO research, studying the renal response to acute COi and/or acute heat stress is in its infancy. Furthermore, no studies have examined the combined effects of these interventions on EPO concentration, and no studies have employed these acute interventions in females to test whether there are any sex differences in EPO concentrations. Accordingly, the extent to which these acute interventions work independently and in combination to increase circulating EPO concentration in females and males is unknown. However, the change in EPO concentration in response to hypoxia is known to be markedly varied, so in addition to characterizing the renal response to the interventions, we sought to examine the influence of iron status parameters and serum concentrations of inflammatory cytokine as potential sources of the variability in EPO concentration for a given stimulus. We hypothesized that 1) COi + HWI would augment increases in serum EPO concentrations, 2) there would be no sex differences in EPO in response to these interventions, and 3) those with the highest concentrations of baseline inflammatory cytokines and transferrin and lowest concentrations of iron and ferritin would have blunted EPO concentrations in response to renal hypoxemia.

Aim 2. Quantify the influence of immune system components (white blood cell counts and cytokine concentrations), iron status parameters, and the presence or absence of a PFO on Hb mass in females and males at sea level.

Even when Hb mass is normalized to lean body mass, there is still a large degree of variability (up to approximately 300grams), even within one sex assigned at birth at a given lean body mass (Goodrich *et al.*, 2020). While data support the role of the immune system and iron

availability in regulating Hb mass, no studies have examined whether the presence or absence of a PFO influences Hb mass and whether it does so indirectly by altering inflammatory cytokine concentrations, white blood cell counts, or iron status parameters. Therefore, the association of PFO presence or absence with Hb mass and the potential reasons by which this intracardiac shunt could explain Hb mass variability is entirely unknown, but data suggests a link between PFO presence, iron deficiency, and stroke risk (Shovlin, 2014; Topiwala *et al.*, 2021). Accordingly, we measured Hb mass and determined whether there were differences associated with various immune system components, iron status parameters, and the presence or absence of a PFO. We hypothesized that 1) those with the lowest iron availability, highest inflammatory cytokine concentrations, and highest white blood cell counts would have the lowest Hb mass and 2) those with a PFO would have lower Hb mass, which would be largely driven by those with a PFO having lower iron availability.

Aim 3. Determine whether two non-decompression SCUBA dive profiles increase pulmonary pressure and resistance. Examine the influence of NO bioavailability and the presence of a PFO on modulating pulmonary pressure post-SCUBA diving. Quantify SCUBA-induced changes in cytokine concentrations and determine the association between cytokines and pulmonary pressure post-SCUBA diving.

Despite decades of research into HPV, there are limited studies examining the pulmonary pressure changes in response to SCUBA diving, and none that have investigated the mechanisms behind the reported increase in pulmonary pressure in humans. Therefore, we used sildenafil to directly increase NO bioavailability to reduce pulmonary pressure post-SCUBA diving. In addition, we measured serum concentrations of inflammatory cytokines pre- and post-dive to quantify their relationships with pulmonary pressure and determined whether the presence of a

PFO altered pulmonary pressure. We hypothesized that 1) there would be an increase in pulmonary artery systolic pressure (PASP) and total pulmonary resistance (TPR) with SCUBA diving that would be reversed by sildenafil treatment; 2) cytokine concentrations would increase post-dive, and those that had the greatest pre- and post-dive concentrations of inflammatory cytokines would have the greatest increases in pulmonary pressure post-SCUBA diving; and 3) the presence of a PFO would not alter pulmonary pressure.

CHAPTER II

REVIEW OF LITERATURE

INTRODUCTION

This review of literature will begin with a discussion of red blood cell and EPO regulation, with particular emphasis on recent studies that examined COi and heat stress. Next there will be an exposition on pulmonary pressure regulation, including a deep examination of HPV and what little is known about pulmonary vascular tone regulation following SCUBA diving. Lastly, there will be a discussion on the three factors that may modulate inter-individual variability in these cardiopulmonary variables, including several immune system components, iron status parameters, and the PFO.

RED BLOOD CELL MASS AND REGULATION OF EPO PRODUCTION

When oxygen delivery is compromised, hypoxia inducible factor (HIF) pathways are stimulated. HIFs are composed of two subunits, an alpha and beta subunit. Both subunits are constitutively expressed, but under normoxic conditions the alpha subunit is tagged for proteasomal degradation, rendering the HIFs inactive. There are 3 known HIF-α variations, and HIF-2α is essential to EPO production (Jelkmann, 2011; Haase, 2013). EPO is produced primarily by the kidney in adult humans, and when there are reductions in oxygen delivery to the kidney, the peritubular fibroblasts in the renal cortex will increase transcription of EPO mRNA (Jelkmann, 2011), resulting in increased EPO protein concentration.

In addition to the kidney, there are extra-renal sites implicated in EPO protein production. These sites may either directly produce EPO or modulate EPO production by the kidney. The liver is the primary site of EPO production during embryonic development and can contribute to adult EPO production (Haase, 2010). However, EPO mRNA has been shown to be expressed in

most other tissue types, including the brain, lungs, heart, bone marrow, spleen, and reproductive tract (Haase, 2010). In addition, the skin has also been shown to modulate EPO production. Chronic hypoxia can be sensed in the epidermis via a HIF-dependent mechanism, and the HIF-dependent increase in NO will alter skin blood flow, which can modulate EPO production (Boutin *et al.*, 2008; Haase, 2010; Nairz *et al.*, 2012). It is important to note that the kidney is still required to sense a reduction in renal perfusion for EPO concentration to increase.

Hypoxia studies consistently show dramatic increases in EPO gene and protein expression, and this response is largely dose dependent (Abbrecht & Littell, 1972; Ge *et al.*, 2002). Notably, however, there is a large degree of variability in EPO concentration in response to hypoxia, with one study showing changes in EPO concentrations in humans ranging from -41 to 400% after 24 hours at 2800m (Ge *et al.*, 2002). Importantly, this was not an abnormal finding, as an additional study reports variability in EPO concentration ranging from an approximate 20% increase to a 200% increase in response to breathing hypoxia (fraction of inspired oxygen of 0.15) for 4 hours (Friedmann *et al.*, 2005). Altitude studies characterized by sustained hypoxia (greater than approximately 48 hours) show that the EPO concentration peaks around 24-48 hours after the onset of altitude exposure (Faura *et al.*, 1969; Abbrecht & Littell, 1972; Milledge & Cotes, 1985; Berglund *et al.*, 2002), whereas acute hypoxic exposure (approximately 2 hours) elicits a peak increase in EPO concentration approximately 4-6 hours post-exposure (Wojan *et al.*, 2021).

When an increase in EPO is sustained beyond a few hours, it can elicit an increase in Hb mass. In addition, the lifespan of red blood cells is approximately 120 days, so approximately $1.7 \cdot 10^{11}$ new red blood cells are made every day to replace those that are no longer functional (Lew & Tiffert, 2017). The process of maintaining Hb mass in the face of high cell turnover is tightly

regulated by EPO, such that EPO is maintained at low concentrations at sea level, with concentrations just sufficient to maintain Hb mass (Jelkmann, 2011).

EPO acts primarily on early erythroid precursors and not on later stage erythroid precursors, reticulocytes, or red blood cells. The distinct erythroid cell line occurs downstream of the common myeloid progenitor with a cell called a BFU-E (Jelkmann, 2011; Dzierzak & Philipsen, 2013; Nandakumar *et al.*, 2016). Downstream of BFU-E cells are CFU-E cells. While not involved in every step of differentiation and maturation, EPO is primarily responsible for the survival of CFU-E cells (Jelkmann, 2011; Nandakumar *et al.*, 2016). EPO is also involved in the differentiation of BFU-E cells to CFU-E cells (Jelkmann, 2011; Nandakumar *et al.*, 2016) (Fig 1).

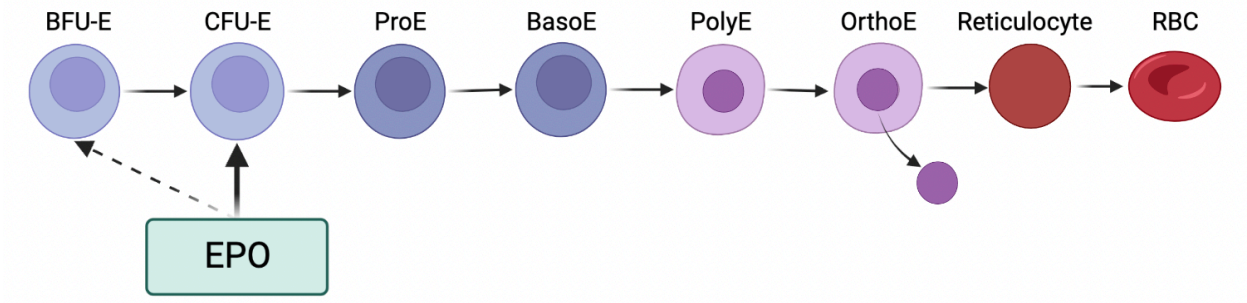


Figure 1. Process of erythropoiesis. EPO primarily acts on CFU-E cells and to a lesser extend BFU-E cells. Later stage erythroid precursors do not express the EPO receptor (EPOR). BFU-E is blast forming unit-erythroid; CFU-E is colony forming unit-erythroid; ProE is proerythroblast; BasoE is basophilic erythroblast; PolyE is polychromatic erythroblast; OrthoE is orthochromatic erythroblast; RBC is red blood cell.

BFU-E and CFU-E cells each express the EPO receptor (EPOR) in high concentrations on their cell surface, allowing them to be highly responsive to circulating EPO. As cells in the

erythroid cell line continue to differentiate and mature, the expression of EPOR diminishes such that later steps in erythropoiesis become EPO independent, and reticulocytes do not express EPOR (Broudy *et al.*, 1991). EPOR is associated with JAK2. When EPO binds its receptor, it activates JAK2, which phosphorylates EPOR and allows for binding of other signaling molecules that contain the SH2 domain (Gillinder *et al.*, 2017; Bhoopalan *et al.*, 2020; Tóthová *et al.*, 2021). The primary signaling pathway involves the activation of STAT5 via its SH2 domain (Haase, 2010; Nairz *et al.*, 2012; Gillinder *et al.*, 2017; Perreault & Venters, 2018; Bhoopalan *et al.*, 2020; Tóthová *et al.*, 2021). Along with JAK2/STAT5 signaling, EPO has also been shown to activate phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (Haase, 2010; Nairz *et al.*, 2012; Perreault & Venters, 2018; Tóthová *et al.*, 2021), but it has been shown that the EPO-JAK2-STAT5 axis is sufficient to induce erythropoiesis (Grebien *et al.*, 2008). Along with STAT5, there are other transcription factors important for erythropoiesis, namely T-cell acute lymphocytic leukemia protein 1 (TAL1), GATA binding factor 1 (GATA1), and erythroid kruppel-like factor 1 (KLF1), which are often termed 'master regulators' (Perreault & Venters, 2018). In one study, EPO stimulation in murine J2E cells resulted in STAT5 occupation on hundreds of genetic locations, and those genes that were co-occupied by the master regulators, GATA1 and KLF1, were linked to genes involved in erythropoiesis (Gillinder *et al.*, 2017).

The downstream targets of the EPO-JAK2-STAT5 axis are numerous and still being elucidated. However, hundreds of downstream targets have been identified (Gillinder *et al.*, 2017; Bhoopalan *et al.*, 2020), and their functions are largely involved in providing negative feedback on EPOR, preventing apoptosis of erythroid cells, promoting erythroid proliferation, as well as various non-erythropoietic functions (Silva *et al.*, 1996; Dolznig *et al.*, 2002; Nairz *et al.*,

2012; Bhoopalan *et al.*, 2020; Tóthová *et al.*, 2021). It is thought that EPO acts on the Fas ligand (FasL) system to prevent apoptosis (Nairz *et al.*, 2012) but also acts through B-cell lymphoma extra-large (Bcl-XL) and B-cell lymphoma 2 (Bcl-2) to prevent apoptosis (Silva *et al.*, 1996; Dolznig *et al.*, 2002). EPO's non-erythropoietic mechanisms include altering immune system function and iron regulation (Nairz *et al.*, 2012; Bhoopalan *et al.*, 2020). In addition to its more well-studied role of increasing Hb mass, EPO has also been implicated in altering the renin-angiotensin-aldosterone axis and therefore regulating plasma volume (Lundby *et al.*, 2007), which is important during early hours of ascent to high altitude.

Despite the relatively rapid increase in EPO when oxygen delivery to the kidney is reduced, it takes approximately 7-14 days for a measurable increase in Hb mass, depending on the strength of the stimulus (Garvican *et al.*, 2012; Ryan *et al.*, 2014). An interesting question, however, is what causes EPO protein concentration to decrease after approximately 48 hours of sustained hypoxic exposure and how Hb mass increases without the consistent EPO stimulus. After only 48 hours, there is no meaningful increase in Hb mass, so this cannot be the negative feedback signal to decrease EPO production. Some have postulated that the increased affinity of Hb for oxygen or the actions of EPO on plasma volume and therefore Hb concentration are more important in negatively regulating EPO (Klausen, 1998; Lundby *et al.*, 2007). In addition, since EPO is involved early in the process of red blood cell differentiation, the delay between the peak in EPO and the increase in Hb mass likely reflects EPO-independent aspects of red blood cell development and maturation.

Even if two individuals have comparable increases in EPO with a given stimulus, this may not translate to the same increase in Hb mass. This is most typically seen clinically, in which chronic kidney disease (CKD) patients may need different doses of EPO stimulating

agents to increase Hb mass despite the same degree of anemia (López-Gómez *et al.*, 2008). Similarly, there are racial differences (Lacson *et al.*, 2008; Kaufman, 2008) and sex differences (Coronado Daza & Cuchi, 2019) in EPO responsiveness in CKD patients. To the best of our knowledge, no studies have specifically looked at the degree of EPO responsiveness by quantifying the strength of the relationship between acute EPO increases and chronic Hb mass increases. What is well known is that to have a measurable increase in Hb mass, EPO concentration must increase, but there are other hormones aside from EPO that can regulate red blood cells and may explain differences in the ability of EPO to increase Hb mass (Lodish *et al.*, 2010). Some of these factors will be discussed in the immune system and iron regulation subsections below. In addition, the variation in EPO stimulating agent dosage can be at least somewhat complicated by potential unmeasured differences in renal blood flow (Q_R). This is an important consideration, as most studies examining EPO and Hb mass only quantify one half of the stimulus, CaO_2, and not Q_R.

As described earlier, the primary function of mature erythrocytes is oxygen delivery. During erythrocyte maturation, cells expel their nucleus and most organelles such that mature erythrocytes are functionally composed of cytoplasm and the protein Hb. Hb is a ferric protein with four subunits, each capable of binding a molecule of oxygen. Of all the oxygen in the blood, the majority of it is carried bound to Hb. In fact, without the oxygen carrying capacity of Hb, resting Q would need to exceed 80 L/min in humans in order to maintain necessary resting oxygen consumption, and Q this high is not physiologically possible in humans (Levitsky, 2007). As Hb binds more oxygen, the affinity of oxygen for Hb increases, a process called cooperativity. Cooperativity is what gives the oxyhemoglobin curve its sigmoidal shape.

Along with oxygen, Hb can also bind other gasses. A small amount of carbon dioxide in the blood is transported by Hb. In addition, CO has an extremely high affinity for Hb, approximately 210 times higher than that of oxygen (Levitsky, 2007). CO therefore has important clinical and research uses, and more recently it has been implicated as a therapeutic tool in a clinical setting (Hess, 2017; Goebel & Wollborn, 2020). Techniques to measure Hb mass rely on the increased binding affinity of Hb for CO by having participants breathe a small volume of CO and measuring changes in HbCO% (the percentage of Hb bound to CO, or carboxyhemoglobin) (Siebenmann *et al.*, 2017). This technique is a central method to the dissertation work presented here. In addition to its use measuring Hb mass, breathing CO results in functional hypoxemia. For this dissertation, COi was used as a form of hypoxemia that only reduced CaO_2 without altering the partial pressure of oxygen in the blood, which avoided stimulating other responses to hypoxia, such as increased ventilation (Dominelli *et al.*, 2019). In addition, unlike breathing air with a reduced fraction of inspired oxygen, the hypoxic stimulus with COi lasts beyond the period of COi, as CO clearance in the blood takes several hours (Zavorsky *et al.*, 2014).

Aim 1 of this dissertation examined EPO concentrations in response to two different forms of decreased renal oxygen delivery, COi and acute heat stress via HWI. The form of hypoxia is an important consideration to any study design. As noted above, COi specifically reduces the amount of Hb bound to oxygen and therefore lowers CaO_2 but does not alter blood oxygen tension. Studies have shown that increased EPO concentration in response to hypoxia is CaO_2 dependent and independent of oxygen tension (Montero & Lundby, 2019). Therefore, COi is an appropriate intervention to increase renal EPO production without stimulating additional effects of hypoxia. Importantly, there are other effects of CO, such as its role as an anti-

inflammatory molecule (Goebel *et al.*, 2008; Hess, 2017; Goebel & Wollborn, 2020) and its inhibition of the carotid body (Prabhakar *et al.*, 1995), which may result in unforeseen systems interactions.

Renal oxygen delivery is a product of Q_R and CaO_2 (*equation 1, "Equations appendix"*). On one hand, COi will decrease renal oxygen delivery by decreasing the amount of Hb bound to oxygen and therefore decrease CaO_2 (*equation 2, "Equations appendix"*). On the other hand, heat reduces oxygen delivery to the kidney by reducing Q_R. At rest, the kidney receives up to 20% of Q, well in excess of its own metabolic demand as an organ (Rowell, 1993). When blood flow needs to be diverted elsewhere during a physiological stressor, such as during exercise or heat stress, blood is diverted from the kidney to areas requiring blood flow, like skin and skeletal muscle. Therefore, the goal of using heat was to reduce the Q being delivered to the kidney (*equation 1, "Equations appendix"*). Importantly, it is possible that COi and heat stress do not exclusively target CaO_2 and Q_R, respectively. COi may also alter Q_R, although this is unknown. As noted above, hypoxia will stimulate HIF, which can increase NO production in the epidermis, and the increased skin blood flow due to NO can reduce renal perfusion and thereby increase EPO (Boutin *et al.*, 2008). Whether COi alters Q_R via this pathway or another unanticipated pathway is unknown. In addition, heat stress can alter CaO_2 by right shifting the oxyhemoglobin dissociation curve, lowering the binding affinity of oxygen for Hb, and thereby reducing CaO_2.

Few studies have examined the effects of COi on EPO production and on Hb mass. However, from these few studies, the stimulus for EPO production and subsequent formation of red blood cells with COi appears to be dependent on the severity and frequency of the stimulus. In two studies, one by Wang *et al.* (2019*a*) and one by Schmidt *et al.* (2020), periodic COi was shown to increase Hb mass chronically. Both studies had participants breathe similar volumes of

CO for a comparable amount of time – Wang *et al.* (2019*a*) had participants breathe 1 mL/kg body mass in 4L of 100% oxygen for 2 minutes, and Schmidt *et al.* (2020) had participants breathe 0.8-1.0 mL/kg body weight in room air for 2 minutes. Additionally, both studies elicited a peak HbCO of approximately 4-7%. The main differences in study design were that Wang *et al.* (2019*a*) had participants breathe CO once daily for 5 days a week prior to exercise training for 4 weeks, whereas Schmidt *et al.* (2020) did not employ exercise but rather had participants breathe CO 5 times daily for 3 weeks to elicit a sustained increase in HbCO. Interestingly, only Wang *et al.* (2019*a*) found a statistically significant 42% increase in serum EPO concentration approximately 4 hours after COi. The likely explanation for the lack of significant increase in EPO from Schmidt *et al.* (2020) is that EPO concentration was measured in the whole group only once weekly, so the peak EPO concentration acutely post-COi may not have been captured. They did measure EPO concentration in a subset of 3 participants every 8 hours for 72 hours post-COi and found that EPO concentration was higher compared to their control group. One last important note from the work conducted by Schmidt *et al.* (2020) is that the increase in Hb mass coincided with a decrease in ferritin concentration, indicating that iron stores were being utilized in the formation of new red blood cells.

Despite those two studies finding COi can increase Hb mass, one other study showed negative results on the effect of COi on Hb mass. Breathing CO (1.2 mL/kg body mass for 30 seconds) once daily for 10 days to raise HbCO to approximately 4.4% elicited no increase in Hb mass (Ryan *et al.*, 2016). Compared to the study by Schmidt *et al.* (2020) in which the reduction in oxygen delivery was continuous and sustained, or the study by Wang *et al.* (2019*a*) in which exercise may have provided an additive stimulus, it is likely that one bolus of CO per day for only 10 days was not a strong enough stimulus. This could be because either the reduction in

renal oxygen delivery was not severe enough, the frequency of intermittent stimulus was not sufficient, or 10 days was not a long enough duration to achieve a measurable increase in Hb mass. It takes approximately two weeks for full erythropoiesis to occur, data which is supported by Schmidt *et al.* (2020). One of the few studies that has shown significant increases in Hb mass in 7 days was at an extreme altitude of 17,260 feet that also involved exercise (Ryan *et al.*, 2014), and the reduction in oxygen delivery achieved by Ryan *et al.* (2016) was not nearly as strong. At 17,260 feet arterial oxygen saturation (SaO_2) was approximately 78% (Subudhi *et al.*, 2014) while the HbCO achieved by Ryan *et al.* (2016) would result in SaO_2 values of approximately 92-95%. Consequently, it seems that in order to have an increase in EPO acutely that results in an increased Hb mass with COi, there either has to be 1) a sustained hypoxic stimulus (Schmidt *et al.*, 2020), 2) additive stimuli which reduce Q_R such as exercise (Wang *et al.*, 2019*a*), or 3) a more severe stimulus (not yet investigated).

Similar to the use of COi, the study of the hematological adaptations to acute and chronic heat stress are in their infancy. Chronic whole body heating has been used to elicit increases in endurance exercise performance in both temperate and hot environments (Scoon *et al.*, 2007; Lorenzo *et al.*, 2010; Buchheit *et al.*, 2011; Racinais *et al.*, 2014; Kirby *et al.*, 2021; Lundby *et al.*, 2023), so there is presumably another adaptation beyond being acclimated to exercise in the heat that is resulting in better exercise performance, which is likely through augmenting Hb mass. This is supported by studies that found that in various endurance athletes, training while in a heated chamber, heated suit, or clothing that prevents heat loss resulted in increases in Hb mass compared to control participants after approximately 5 weeks (Oberholzer *et al.*, 2019; Rønnestad *et al.*, 2021, 2022; Lundby *et al.*, 2023). Interestingly, Rønnestad *et al.* (2021, 2022) did not see an increase in VO_2max despite an increase in Hb mass with chronic heating, but it is

important to note that both studies employed elite athletes who may have had an exercise performance limitation unrelated to Hb mass. Despite chronic whole-body heating increasing Hb mass and endurance exercise performance, two studies showed that EPO did not increase after acute heat stress (Akerman *et al.*, 2017; Oberholzer *et al.*, 2019). However, similar to data obtained by Schmidt *et al.* (2020) on the effect of COi on EPO concentration, the negative results by Oberholzer *et al.* (2019) could be a result of only measuring EPO once after the intervention and not capturing the 4-6 hour window in which EPO increases acutely post-intervention (Wang *et al.*, 2019*a*; Wojan *et al.*, 2021). However, Akerman *et al.* (2017) did not find an increase in EPO 2 hours post-heat stress, so exploration of the acute mechanisms by which heat stress works to chronically stimulate an increase in Hb mass is needed to reconcile the contradiction between the studies showing heat stress does not increase EPO but does increase Hb mass and exercise performance.

An additional factor considered in this dissertation, primarily in **Aim 1** and to a lesser extent **Aim 2**, is the role of sex assigned at birth. It is well documented that females have significantly lower Hb mass than males when expressed as absolute grams and when normalized to body mass (Goodrich *et al.*, 2018; Falz *et al.*, 2019). Females also have lower Hb concentration (Murphy, 2014; Handelsman *et al.*, 2018). Additionally, regardless of the method used to quantify lean body mass (the gold standard dual x-ray absorptiometry or bio-impedance analysis), females have lower Hb mass when normalized to lean body mass (Falz *et al.*, 2019; Goodrich *et al.*, 2020). However, Goodrich *et al.* (2020) found that the group means between females and males were closer when comparing Hb mass normalized to lean body mass compared to when they normalized Hb mass to whole body mass.

Red blood cells can be regulated by sex hormones, which likely accounts for much of the sex differences in Hb mass observed. Testosterone stimulates red blood cell production. It does so by stimulating EPO production as well as increasing the sensitivity of erythroid precursor cells to EPO, and testosterone can also suppress hepcidin, which increases iron availability for production of red blood cells (Shahani *et al.*, 2009; Bachman *et al.*, 2014). Females have lower testosterone and therefore do not get the stimulatory effects of this hormone on Hb mass. In addition, female sex hormones can be inhibitory towards the formation of red blood cells by inducing apoptosis (Dukes & Goldwasser, 1961; Blobel & Orkin, 1996). Despite females having lower Hb concentration and Hb mass, there is not an increased drive for erythropoiesis in females, indicating sex differences in the set point of this response (Murphy, 2014). Interestingly, Hb mass normalized to body mass was found to be higher in females using oral contraceptives compared to females not using oral contraceptives (Keller *et al.*, 2020), which may point to differences in red blood cell regulation by synthetic versus natural sex hormones or to the relative concentrations of sex hormones.

Importantly, even though lean body mass has the strongest correlation with absolute Hb mass, for a given lean body mass, Hb mass can range approximately 200-300 grams, even within the same sex assigned at birth (Goodrich *et al.*, 2020). A change in Hb mass of only 34 g elicited a significant increase in VO_2max of 1.6 mL/kg/min (Wang *et al.*, 2019*a*), so it is likely that a 300 gram range of Hb mass is physiologically meaningful as it pertains to oxygen delivery and exercise performance. Reasons for the individual variability independent of lean body mass will be explored in **Aim 2** of this dissertation and discussed in the iron, immune system, and PFO subsections of this literature review below.

In contrast to Hb mass, there are no differences in reference baseline values for EPO between females and males (Murphy, 2014). As noted above, there may be sex differences in EPO responsiveness (Coronado Daza & Cuchi, 2019), at least in CKD patients, but no data exists to support sex differences in baseline EPO concentrations or EPO concentrations in response to hypoxia in healthy participants. However, there are sex differences in immune function and iron regulation, which may result in sex-based differences in EPO concentration in response to hypoxia. Sex differences in immune function and iron regulation and how that impacts EPO concentrations will be discussed in more detail in the appropriate sections below.

REGULATION OF PULMONARY VASCULAR TONE

Compared to systemic circulation, pulmonary arteries are characterized by relatively less muscle and neural control of vascular tone. Because of the minimal neural control, the pulmonary arteries are largely controlled by local mechanisms and physical forces. At resting sea level conditions, pulmonary arteries have little to no active tone. This contrasts with systemic circulation in which some degree of resting vascular tone exists, allowing vessels to either dilate or constrict in response to stimuli like exercise, hypoxia, heat, or vasoactive molecules. When Q rises, to keep mean pulmonary artery pressure low, the pulmonary vessels will passively distend, and under-perfused and non-perfused vessels will be recruited. That is, not all pulmonary vessels are recruited and receive blood flow at rest. However, despite the largely passive nature of the pulmonary circulation, there are instances in which pulmonary vascular tone is actively rather than passively regulated. Notably, hypoxia will cause an active vasoconstriction of pulmonary arterioles, termed hypoxic pulmonary vasoconstriction (HPV), which can result in an increase in pulmonary pressure if the lung hypoxia is global. In addition, SCUBA diving during

decompression dives has been shown to increase pulmonary pressure (Dujić *et al.*, 2006; Marabotti *et al.*, 2013).

The increase in pulmonary pressure is seen post-SCUBA diving when oxygen saturation is normal (Dujić *et al.*, 2006; Marabotti *et al.*, 2013). Unlike high altitude, SCUBA diving is a hyperbaric condition and therefore a hyperoxic environment, so the mechanisms underlying this response remain unclear. Studies have shown that there is redistribution of pulmonary blood flow with hyperoxia (Melsom *et al.*, 1999; Hlastala *et al.*, 2004), and this active redistribution of blood flow suggests vasoconstriction may be at play. In addition, acute hyperoxia during an altitude expedition does not reduce pulmonary pressure (Groves *et al.*, 1987), indicating that hyperoxia may actively regulate pulmonary pressure and does not simply shut off HPV. Another possible explanation for increased pulmonary pressure post-SCUBA diving is redistribution of blood flow related to immersion and hyperbaria. The hyperbaria and reduced effects of gravity during immersion will increase pulmonary blood volume and therefore pressure (Moon *et al.*, 2009). However, pulmonary pressure is reported to remain elevated hours after a dive has occurred (Marabotti *et al.*, 2013) when hyperbaria and hyperoxia, redistribution of pulmonary blood flow, etc. is no longer a factor. Therefore, the increase in pulmonary pressure is not simply related to the effects of water immersion or hyperoxia.

Pulmonary pressure can increase due to either an increase in Q or pulmonary resistance, or both. Studies that show increased pulmonary pressure post-SCUBA diving fail to distinguish between the contribution of increased TPR and Q (Dujić *et al.*, 2006; Marabotti *et al.*, 2013), so whether SCUBA diving results in pulmonary vasoconstriction is unknown. However, because Marabotti *et al.* (2013) found pulmonary pressure to be elevated two hours post-dive when any changes in Q due to water immersion or exercise would no longer be a factor, it is presumed that

a sustained vasoconstriction is responsible for the increase in pulmonary pressure post-SCUBA diving.

While one focus of this dissertation is on regulation of pulmonary vasculature during SCUBA diving, I will spend a portion of this literature review discussing the mechanisms of HPV. The mechanisms causing an increase in pulmonary pressure post-SCUBA diving are unknown, but both SCUBA diving and hypoxia are characterized by an active increase in pulmonary pressure. Therefore, despite SCUBA diving being a hyperoxic environment, some of the mechanisms responsible for governing vascular tone during hypoxia may be at play during and/or post-SCUBA diving as well.

HPV occurs in response to a combination of alveolar hypoxia and pulmonary arterial hypoxemia and can occur throughout the entire lung or regionally within the lung. The benefit of this response is particularly notable during regional lung hypoxia. In this context, the pulmonary vessels surrounding the local hypoxic region of the lung can constrict, which will divert blood flow to other regions of the lung to ensure better oxygenation via improved ventilation to perfusion matching. In this context, improving ventilation perfusion matching has a positive impact on oxygen delivery by improving arterial PO_2 (PaO_2). HPV can be detrimental in the context of whole lung hypoxia, such as that which occurs during high altitude exposure. During whole lung hypoxia, there are no areas of the lungs that are better ventilated to divert blood flow to, so the global vasoconstriction results in an increase in pulmonary pressure and does not improve oxygenation of the blood to the same degree as it does with regional alveola hypoxia. This increase in pressure can vary drastically among individuals despite the same stimulus (Grünig *et al.*, 2000; Swenson, 2013), so understanding the mechanisms regulating pulmonary

vascular tone post-SCUBA diving may explain individual variability and help elucidate mechanisms governing pulmonary vascular tone in hyperoxic environments.

HPV has been shown to occur in isolated pulmonary artery smooth muscle cells (Madden *et al.*, 1992), so this response is primarily driven by the smooth muscle itself. Within the smooth muscle cells, oxygen-dependent potassium channels are inhibited during hypoxia, resulting in membrane depolarization and calcium influx, causing muscle contraction (Post *et al.*, 1992; Weir & Archer, 1995; Sommer *et al.*, 2008). While hypoxia can inhibit multiple potassium channels, HPV is driven by inhibition of the Kv1.5 channel specifically (Archer *et al.*, 2001). The potassium and calcium channels are necessary for HPV to occur, as evidenced by a diminished HPV with calcium channel blocking (McMurtry *et al.*, 1976) and an augmented HPV with potassium channel activation (Hasunuma *et al.*, 1991). The primary oxygen sensors for HPV are mitochondria, which are responsible for producing reactive oxygen species (ROS). The ROS are then responsible for the potassium channel inhibition, initiating HPV (Archer *et al.*, 1993; Weir *et al.*, 2005; Sommer *et al.*, 2008; Schumacker, 2011; Dunham-Snary *et al.*, 2017).

In addition to the changes in ion currents that drive HPV, there are vasoactive substances that may be modulating pulmonary vascular tone, including but not limited to NO, endothelin-1, ATP, and inflammatory cytokines. Evidence suggests that hypoxic production of endothelin-1 may play a role in HPV (Oparil *et al.*, 1995), partially by directly inhibiting potassium currents (Shimoda *et al.*, 1998). However, historically, vasoactive substances were viewed as moderators rather than primary regulators of HPV due to the ability of isolated pulmonary artery smooth muscle cells to contract in the absence of intact endothelium (Weir & Archer, 1995). More recent evidence, particularly in intact humans, suggests larger roles for vasoactive substances.

Importantly, there is a biphasic HPV response, with an initial increase in pulmonary pressure occurring in the first few minutes of hypoxic exposure, followed by a delayed, greater increase within a few hours (Talbot *et al.*, 2005; Sylvester *et al.*, 2012; Swenson, 2013). It is thought that the phase I initial increase in pulmonary pressure is due directly to the actions of the pulmonary artery smooth muscle cells and inhibition of potassium channels, while the delayed phase II increase is a result of additive modulating substances, such as NO and endothelin availability, inflammation, or erythrocyte byproducts (Aaronson *et al.*, 2002; Sylvester *et al.*, 2012; Swenson, 2013). This is seen by a reduced phase II constriction in pulmonary arteries without intact endothelium (Aaronson *et al.*, 2002). Given the time delay and sustained increase in pulmonary pressure seen post-SCUBA diving, it is reasonable to speculate that the mechanism for the increase in pulmonary pressure may be largely driven by vasoactive substances similar to those that regulate the phase II HPV response. **Aim 3** used sildenafil to increase NO availability, which is a potent vasoactive substance that may play a role in pulmonary pressure regulation post-SCUBA diving. In addition, inflammatory cytokine concentrations were analyzed pre- and post-dive as modulators of pulmonary pressure, and their potential role in regulating pulmonary vascular tone will be discussed further in the immune system subsection of this literature review.

NO is a potent vasodilator, both in the systemic and pulmonary circulations. Under normoxic conditions, NO plays a role in maintaining resting pulmonary vascular tone (Cooper *et al.*, 1996). Additionally, under hypoxic conditions, studies have shown that inhibition of NO synthase, and therefore reduced NO availability, potentiates HPV in humans (Blitzer *et al.*, 1996). However, there are other studies that show no role of NO in HPV in *in vitro* models (Aaronson *et al.*, 2002). Despite the negative results found by Aaronson *et al.* (2002), studies in intact humans showed that HPV was NO dependent (Blitzer *et al.*, 1996; Bailey *et al.*, 2010).

The reason for this discrepancy is unclear, but the NO dependence in human studies led us to believe that NO may play a role in the SCUBA diving increase in pulmonary pressure. Studies have used sildenafil as a treatment for pulmonary hypertension, particularly in those forms of pulmonary hypertension secondary to lung diseases like COPD where HPV may be a factor and NO may play a role (Barnes *et al.*, 2019). Additionally, sildenafil has been shown to reduce pulmonary pressure with hypoxia (Carter *et al.*, 2019; Kelly *et al.*, 2022) and mean pulmonary artery and wedge pressure with immersion pulmonary edema (Moon *et al.*, 2016; Martina *et al.*, 2017). Therefore, NO is a significant vasoactive molecule involved in regulating pulmonary vascular tone, and it may play a role regulatory role during the changes in pulmonary vascular tone post SCUBA diving as well.

IMMUNE SYSTEM

Overview

The human immune system can be divided into the innate and adaptive immune systems. The combination of these two systems allows the human immune system to have very rapid, strong, specific, and tailored responses to a foreign pathogen (virus, bacteria, etc.), foreign benign particle (such as dust, or another allergen), or even a benign non-self-cell (such as would occur during an organ transplant). Immune cells, commonly called white blood cells, are part of the hematopoietic stem cell (HSC) lineage. The two main cell lines that arise from HSCs are the lymphoid cells, which primarily make up our adaptive immune system, and myeloid cells, which compose our innate immune system. The myeloid cell line also gives rise to red blood cells (erythrocytes), as discussed above. For this literature review, we will discuss cells downstream of the HSC cell line as being myeloid, lymphoid, or erythroid cell lines.

Cells of the myeloid cell line consist of the general classes of granulocytes and phagocytes. Types of granulocytes include neutrophils, basophils, and eosinophils. Macrophages, monocytes, and dendritic cells are the main types of phagocytes. Within the lymphoid cell line, cell types include T cells (cytotoxic and helper T cells), B cells, and natural killer cells. Phagocytes are important for antigen presenting as well as phagocytosis. They will engulf foreign particles or pathogens and digest those proteins, as well as recycle old or senescent cells. Additionally, phagocytes will present antigen peptides on their surface, which is important in initiating an adaptive immune response. Neutrophils also have phagocytotic capabilities and can cause direct harm to pathogens. Basophils help regulate the adaptive immune response and are implicated in inflammation as well as vasodilation and smooth muscle activation via histamine contained in the basophils (Owen *et al.*, 2013). Lastly, eosinophils are anti-viral & phagocytic and help modulate the adaptive immune response (Owen *et al.*, 2013). The main function of B cells is to secrete antibodies specific to pathogen peptides. Helper T cells will mainly work by secreting cytokines to help tailor B cell and cytotoxic T cell responses. Cytotoxic T cells are responsible for killing pathogens.

Cytokines and chemokines are cellular messenger proteins that modulate the immune response and mobilize the immune cells discussed above. Cytokines are released by immune cells but can also be released by epithelial cells and smooth muscle cells. They can have endocrine, paracrine, or autocrine functions, and the effector cell must have a receptor for the cytokine to respond. Cytokines have a wide range of functions and generally help either suppress or activate the immune system, or in some instances both. Chemokines are similar proteins, but their main function is initiating cell migration. Importantly, cytokines and chemokines rarely act alone and are all released in an inflammatory cascade. In addition, the human immune system is

characterized by redundancy and pleiotropy. Redundancy describes the process by which multiple cytokines and immune cells share the same function, and pleiotropy is the process by which one cytokine or immune cell has numerous functions. Therefore, it is difficult to determine the functions of individual cytokines in an immune response in an intact human model.

There are many classes of cytokines. Some are characterized by similarities in function, while some are characterized primarily by structural similarities and have a wide range of functions. For this dissertation, the following cytokines and chemokines were studied: Interleukins (IL)-1β, 6, 8, 10, 12p70, 17A, 18, 23, and 33; interferons (IFN)-α2 and γ; tumor necrosis factor (TNF)-α; and monocyte chemoattractant protein (MCP)-1. These cytokines and chemokines were selected due to both their known roles in the areas of research we examined and the lower costs of using a pre-defined multiplex assay instead of many single cytokine immunoassays for protein concentration quantification.

The IL-1 family consists of IL-1β, IL-18, and IL-33, and these cytokines are responsible for initiating a robust pro-inflammatory response (Arend *et al.*, 2008; Owen *et al.*, 2013). These cytokines are typically produced by monocytes or macrophages early in the immune response, and they can stimulate the production of other cytokines (Owen *et al.*, 2013). In addition, IL-33 is expressed in smooth muscle cells (Owen *et al.*, 2013), which will become important in the discussion below on regulation of pulmonary vascular tone.

IL-6, IL-12p70, and IL-23 fall into the hematopoietin family, which has a large range of functions. EPO falls into this category as well and can act as an anti-inflammatory cytokine (Nairz *et al.*, 2012). When elevated concentrations of EPO are sustained, it promotes the differentiation of hematopoietic cells to the erythroid cell line, thus minimizing the number of

white blood cell precursors (Paulson *et al.*, 2020). With fewer white blood cells, there is likely to be less cytokine release, which may partially explain the anti-inflammatory effects of EPO. IL-12p70 and IL-23 are known to induce the generation of two subsets of T helper cells, Th1 and Th17 cells respectively, which promote inflammation (van der Heijden *et al.*, 2019). IL-6 is primarily involved in stimulation of B cells, but it has a large range of functions. It is considered both pro- and anti-inflammatory depending on the context, and it is secreted by numerous cell types, including endothelial cells (van der Heijden *et al.*, 2019). While it can induce inflammation and activate the immune system, its release, particularly during exercise, can also help suppress a downstream inflammatory cascade by inhibiting TNF-α and inducing IL-10 expression (Petersen & Pedersen, 2005*a*).

The interferon family consists of IFN-α2, IFN-γ, and IL-10, although more recent studies characterize IL-10 in its own cytokine family (Rutz & Ouyang, 2016). IFN-γ is the most potent activator of the Th17 subset of cells. IL-10 is considered one of the most potent anti-inflammatory cytokines and will suppress the immune system. It is often released in response to other inflammatory cytokines to suppress excessive inflammation (Rutz & Ouyang, 2016). However, it has also been shown to have pleiotropic effects that are largely dependent on the microenvironment and co-release of other cytokines and can promote cytotoxic T cell and B cell functions (Bedke *et al.*, 2019).

TNF-α falls into the tumor necrosis factor family and is a pro-inflammatory cytokine. IL-17A is part of the interleukin 17 family, which is also pro-inflammatory (Gaffen, 2009). Lastly, IL-8 and MCP-1 fall into the chemokine family. IL-8 is essential to the recruitment of neutrophils during an LPS stimulated immune challenge (Harada *et al.*, 1994), and given its name, MCP-1 is implicated in monocyte recruitment during immune challenges.

Non-pathogenic immune system activation & sex differences

Notably, the immune system can be activated in situations where there is no pathogen present, such as during SCUBA diving. SCUBA diving has been shown to increase circulating granulocytes (Marabotti *et al.*, 1999), increase neutrophil counts and activation (Nossum *et al.*, 2002; Thom *et al.*, 2013; Madden *et al.*, 2014), increase inflammatory cytokine expression (Žarak *et al.*, 2021; Rocco *et al.*, 2021), and upregulate genes and mRNA related to the immune response (Eftedal *et al.*, 2013; Sureda *et al.*, 2014). Importantly, SCUBA diving induces changes to the immune system in both decompression and non-decompression dive profiles. Of importance to this dissertation, Žarak *et al.* (2021) showed increases in IL-6 post-dive in each of 5 repeat dives to 20-30 meters for 30 minutes separated by one week, data which is supported by the increase in IL-6 observed by Rocco *et al.* (2021) in which divers completed a decompression dive profile of 50 m for 20 minutes. Therefore, SCUBA diving is sufficient to induce changes in the expression of immune cells and cytokines, both during decompression and non-decompression dives. In addition to SCUBA diving, exercise can induce changes in inflammation. IL-6 increases immediately post-exercise, which will then initiate an anti-inflammatory cascade downstream (Petersen & Pedersen, 2005*a*). It is thought that the release of IL-6 post-exercise and the subsequent suppression of pro-inflammatory cytokines is responsible for the anti-inflammatory benefits of chronic exercise (Petersen & Pedersen, 2005*a*). Depending on the intensity of swimming during a dive, it is possible that the compounded effects of exercise and SCUBA diving should be considered.

Additionally, there are known sex differences in immune cell expression and function. Generally, females mount a stronger immune response to a pathogen (Spitzer, 1999; Klein & Flanagan, 2016). Males will have greater IL-10 production (Torcia *et al.*, 2012), whereas females

have higher neutrophil and macrophage numbers and activity (Bain & England, 1975; Bain, 1996; Spitzer, 1999). It is thought that the sex differences originate from both the influence of sex hormones as well as genes encoded on the X-chromosome (Pennell *et al.*, 2012). Importantly, it has been shown that there are immune fluctuations related to menstrual cycle phase, largely driven by differences in sex hormone expression throughout the menstrual cycle (Oertelt-Prigione, 2012). Studies have reported an elevation of IL-1 and T cells during the follicular phase, while the luteal phase is characterized by elevated TNF-α and monocyte count and a decrease in T cell count (Oertelt-Prigione, 2012). There are also age-related differences in immune cell expression in females, which are thought to be due to changes in sex hormones post-menopause (Chen *et al.*, 2016). One last consideration is that immune cell expression can be altered by contraceptive use, with females on oral contraceptives showing higher counts of neutrophils and leukocytes during both menstrual cycle phases than females not using oral contraceptives (Timmons *et al.*, 2005). Therefore, sex differences in immune system activity may be partially responsible for sex differences in Hb mass and/or EPO concentrations, if any sex differences in these variables exist.

Immune system, red blood cell, and EPO interactions

Along with EPO, there are numerous other cytokines, transcription factors, and additional variables that can play a secondary role in regulating red blood cell differentiation, maturation, and survival (Jelkmann, 2011; Dzierzak & Philipsen, 2013; Nandakumar *et al.*, 2016). Inflammatory cytokines have negative effects on erythropoiesis and therefore Hb mass (Cooper *et al.*, 2003; Nandakumar *et al.*, 2016). When circulating inflammation causes a clinically significant reduction in Hb mass it is termed anemia of inflammation and presents a situation in which Hb mass may be compromised by overactivity of the immune system (Fraenkel, 2017;

Weiss *et al.*, 2019; Ganz, 2019). Anemic hemodialysis patients that respond poorly to erythropoietic stimulating agents were found to have higher expression of various cytokines, including TNF-α and IFN-γ, emphasizing that inflammation can create a disconnect between EPO signaling and production of new red blood cells (Cooper *et al.*, 2003). Similarly, TNF-α, IL-6, and IL-8 were found to be elevated in anemic patients (Keithi-Reddy *et al.*, 2008), and treatment with IL-12 can result in anemia (Dybedal *et al.*, 1995).

The immune system can affect Hb mass via numerous pathways, including altering EPO production in response to hypoxia, impairing proliferation and/or differentiation of HSCs and erythroid precursors, or influencing the lifespan and recycling of mature red blood cells. In addition, when the immune system becomes activated and hematopoietic cells are driven towards myeloid and lymphoid cell lines, the erythroid cell line is inhibited (Paulson *et al.*, 2020). The immune system can also regulate iron availability, particularly through the actions of IL-6, but those pathways will be discussed in more detail in the iron regulation section below. A summary of these pathways is provided below in Figure 2, and a detailed discussion of how cytokines can alter Hb mass via these pathways is discussed in the following paragraphs.

Another important consideration to this discussion is the microenvironment in question. Some cytokines may act differently depending on whether they are expressed in the bone marrow, spleen, or in circulation, and some cytokines may have seemingly contradictory roles in modulating Hb mass depending on whether they're acting on EPO, HSCs, or other erythroid precursors. An additional complicating factor to this discussion is a phenomenon called stress erythropoiesis. While the focus here is on inflammation-induced inhibition of steady state erythropoiesis in the bone marrow, inflammation can also promote stress erythropoiesis in the

spleen. This may be partly responsible for the different actions of cytokines in different microenvironments.

IL-1β, TNF-α, IFN-γ, and IL-6 have all been shown to affect EPO concentrations in response to hypoxia (Fandrey & Jelkmann, 1991; Faquin *et al.*, 1992; Vannucchi *et al.*, 1994; La Ferla *et al.*, 2002). The addition of IL-1β, TNF-α, and IFN-γ to hypoxic cell cultures has been shown to directly inhibit EPO production (Fandrey & Jelkmann, 1991; Faquin *et al.*, 1992; Vannucchi *et al.*, 1994; La Ferla *et al.*, 2002). Additionally, when IFN-γ was co-cultured with IL-1 and TNF-α, it was found to have an additive and synergistic effect on EPO production, respectively (Vannucchi *et al.*, 1994). The mechanism by which both IL-1β and TNF-α impair EPO production has been shown to be a result of increased GATA2 and NF-kB activity downstream of the cytokines (La Ferla *et al.*, 2002). Conversely, in the presence of hypoxia, IL-6 can stimulate EPO production, a contrast from the other pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ (Faquin *et al.*, 1992; Vannucchi *et al.*, 1994). When IL-6 is co-cultured with pro-inflammatory cytokines, the effect is additive such that IFN-γ, IL-1β, and TNF-α all reduce the degree of IL-6 dependent EPO production (Faquin *et al.*, 1992; Vannucchi *et al.*, 1994).

Nearly all cytokines of interest in this dissertation have been found to affect the survival, proliferation, or function of various erythroid precursors ranging from HSCs to later stage erythroblasts. At the level of HSCs, cytokines like IL-1, IFN-γ, and TNF-α largely act by driving the differentiation of HSCs into the myeloid cell line and away from the erythroid cell line (Xiao *et al.*, 2002; Libregts *et al.*, 2011; Pietras *et al.*, 2016; Etzrodt *et al.*, 2019). Specifically, both IFN-γ and TNF-α upregulate a transcription factor called PU.1, which promotes HSC differentiation into white blood cells and opposes the differentiation into erythroid precursors, thereby negatively regulating Hb mass (Libregts *et al.*, 2011; Rothenberg *et al.*, 2019; Etzrodt *et*

al., 2019). In addition, IL-1 can reduce the self-renewal capacity of HSCs (Pietras *et al.*, 2016). However, unlike IL-1, IFN-γ, and TNF-α, which largely act on HSCs by preferentially driving their differentiation away from the erythroid cell line, IL-17 induces the proliferation of HSCs (Mojsilović *et al.*, 2015). However, even promoting proliferation of HSCs could have a negative effect on Hb mass because the HSCs may still be driven towards the myeloid cell line with the addition of other cytokines. The role of IL-8 on HSCs is less clear. Some studies find IL-8 mediated inhibition of HSCs (Lu *et al.*, 1993), while others show IL-8 mediated proliferation of HSCs (Corre *et al.*, 1999). The action of IL-8 is likely dose dependent, with lower doses of IL-8 stimulating HSCs and higher doses of IL-8 inhibiting them. However, work by Hermouet *et al.* (2002) emphasizes that IL-8 is elevated in serum and bone marrow plasma of polycythemia vera and idiopathic erythrocytosis patients, which would suggest a role of IL-8 in promoting the erythroid cell line. As noted above, IL-8 is also slightly elevated in anemic patients. Given the elevation of IL-8 in both anemia (Keithi-Reddy *et al.*, 2008) and polycythemia (Hermouet *et al.*, 2002), the role of IL-8 on erythropoiesis is not completely understood. Likely, it depends on the disease state and microenvironment. The role of IL-8 in a healthy human model is still largely unknown.

In addition to acting on HSCs, many cytokines will act downstream of HSCs on early erythroid precursors. IFN-α and IFN-γ can both inhibit BFU-E cell survival and promote apoptosis (Mamus *et al.*, 1985; Raefsky *et al.*, 1985; Tarumi *et al.*, 1995), and at higher concentrations IFN-γ can suppress CFU-E cells as well (Wang *et al.*, 1995). IFN-γ has been shown to increase cell surface expression of Fas in erythroid precursors, resulting in apoptosis (Dai *et al.*, 1998), and it can upregulate members of the TNF family, which suppress erythroid growth and differentiation (Felli *et al.*, 2005). Similarly, IL-1β, IL-6, and IL-33 have all been

shown to inhibit the proliferation of and suppress the maturation of erythroid precursors (Schooley *et al.*, 1987; McCranor *et al.*, 2014; Swann *et al.*, 2020). Swann *et al.* (2020) found that HSCs and downstream erythroid precursors highly expressed the receptor for IL-33 and that IL-33 was necessary for inflammation induced inhibition of erythropoiesis in the bone marrow. In addition, when they treated their mice with exogenous IL-33, the healthy mice decreased Hb expression and subsequently developed anemia. Lastly, these authors targeted the pathway by which IL-33 suppresses erythropoiesis and found it was NF-kB dependent (Swann *et al.*, 2020).

IL-10 and IL-12 are both interesting cytokines with seemingly contradictory actions on erythropoiesis. IL-10 treatment can inhibit BFU-E formation in peripheral mononuclear blood cells derived from patients with polycythemia vera (Geissler *et al.*, 1998) and healthy patients (Oehler *et al.*, 1999), and IL-12 was found to be expressed at higher concentrations in anemic patients (Dybedal *et al.*, 1995), suggesting that both of these cytokines inhibit erythropoiesis. However, co-culturing IL-10 with EPO was found to stimulate erythroid precursors, and similarly co-culturing IL-12 with EPO and IL-4 was found to stimulate erythroid precursors (Dybedal *et al.*, 1995; Wang *et al.*, 1996). In addition, IL-10 treatment has been shown to reduce inflammation and hepcidin and therefore improve anemia of inflammation (Huang *et al.*, 2017), and the addition of exogenous IL-12 prevented anemia in mice with malaria (Mohan *et al.*, 1999) and positively regulated the growth of HSCs (Jacobsen *et al.*, 1993). Therefore, it seems that IL-10 and IL-12 can both suppress and augment erythropoiesis. The reason for these contradictory results is unknown but perhaps related to the necessity for EPO and/or other cytokines to be present, such that IL-10 and IL-12 may have a stimulatory role on erythropoiesis only when combined with other proteins. However, this is an example of why it is crucial to study the effects of these cytokines in an intact model when investigating physiologically relevant

outcomes. As emphasized above, cytokines are rarely released alone and *in vivo* act on, and respond to, other cytokines. Despite the above studies pointing to IL-12 stimulating erythropoiesis *in vitro*, when IL-12 was administered *in vivo*, it suppressed erythropoiesis by inducing the expression of IFN-γ (Eng *et al.*, 1995). Therefore, the role of IL-12 in erythropoiesis is largely dependent on the model being used and the co-release of other cytokines.

Similar to IL-10 and IL-12, the role of IL-17 depends on what erythroid precursors are in question. As noted above, IL-17 induces HSC proliferation. In addition, IL-17 promotes BFU-E development, but it inhibits CFU-E development (Krstic *et al.*, 2012; Mojsilović *et al.*, 2015). The effects of IL-17 are dependent on the microenvironment since IL-17 has a greater stimulation on erythroid precursors in the spleen than the bone marrow (Mojsilović *et al.*, 2015). To further emphasize the importance of the microenvironment, IL-17 can promote CFU-E cell development in the spleen even while it inhibits CFU-E development in the bone marrow (Mojsilović *et al.*, 2015). However, similar to other cytokines, when IL-17A and EPO are added together to an *in vitro* cell model, IL-17 further enhances the proliferative effects of EPO on erythroid precursors (Hwang *et al.*, 2017) even though it may play an inhibitory role on some erythroid precursors without the addition of EPO.

Lastly, some cytokines are involved in the regulation of later stage erythroblasts and mature red blood cells. IL-33 is necessary for the development of splenic red pulp macrophages, which are responsible for recycling red blood cells (Lu *et al.*, 2020; Xu & Huang, 2020). Therefore, when IL-33 expression is chronically elevated, it may promote increased recycling of red blood cells. Additionally, IFN-γ can reduce the lifespan of red blood cells (Libregts *et al.*, 2011). IL-6 has also been shown to suppress genes involved later in erythropoiesis and impair Hb synthesis (McCranor *et al.*, 2014). However, the actions of IL-6 on both early and late-stage

erythroid precursors appears to directly contradict the actions of IL-6 on EPO concentration, such that IL-6 can stimulate EPO production but inhibit erythroid precursors. What ultimate role IL-6 plays in regulating Hb mass in healthy humans is unknown despite its implication in anemia of inflammation (Keithi-Reddy *et al.*, 2008).

The roles of the other cytokines being examined in this dissertation on erythropoiesis are less clear. There have been no direct studies examining the role of MCP-1, IL-18, or IL-23 on erythropoiesis, but based on their general functions we can speculate as to how they may regulate erythropoiesis. For example, EPO can upregulate MCP-1 in human umbilical vein endothelial cells and human vascular smooth muscle cells (Desai *et al.*, 2006), so perhaps EPO increases MCP-1, which then promotes inflammation and monocyte recruitment and serves as a negative regulator of erythropoiesis. Additionally, similar to many pro-inflammatory cytokines, IL-18 was found to be elevated in aplastic anemia patients (Wu *et al.*, 2019). Despite the elevation of IL-18 in these patients, IL-18 deletion in donor lymph node cells did not reduce concentrations of cytokines downstream of IL-18, indicating that IL-18 is likely part of a general immune response and not a direct regulator of aplastic anemia (Wu *et al.*, 2019). However, IL-18 can induce the expression of IFN-γ (Okamura *et al.*, 1995; Wu *et al.*, 2019), so *in vivo* it may play a negative regulatory role on erythropoiesis indirectly through activating IFN-γ. Lastly, given that IL-23 shares the IL-12p40 subunit with IL-12 (Ong'echa *et al.*, 2008), it may act via a similar mechanism to IL-12. Children with malarial anemia were found to have higher circulating IL-23 (Ong'echa *et al.*, 2008), though higher IL-23 has not been shown in anemia of inflammation. IL-23 can increase the expression of other pro-inflammatory cytokines like TNF-α and IL-17 (Pastor-Fernández *et al.*, 2020), so it may indirectly alter erythropoiesis by its actions on those cytokines.

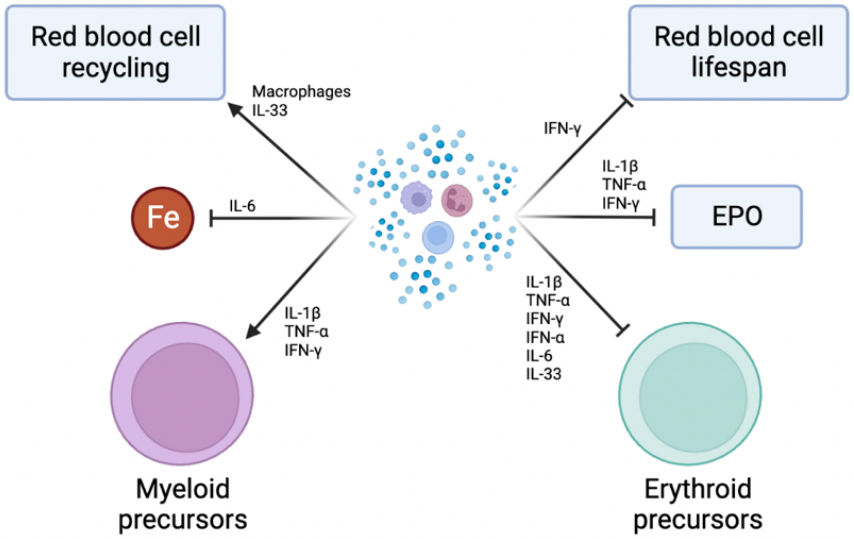


Figure 2. Summary of the pathways through which the immune system can modulate Hb mass. Cytokines and immune cells can negatively regulate Hb mass via numerous pathways, including promotion of red blood cell recycling, reducing red blood cell lifespan, inhibiting iron availability for Hb synthesis, inhibiting EPO production in response to hypoxia, promoting the myeloid cell line, and inhibiting the erythroid cell line. The cytokines and immune cells listed have a known function in each of these pathways, summarized above.

Overall, these studies emphasize a few key points. First, we have limited knowledge on how these cytokines affect Hb mass and EPO concentrations in healthy humans. The studies that use *in vivo* human models mostly show an association of elevated cytokine concentrations with anemia. Other studies use cell culture models or animal models to show how these cytokines target specific erythroid precursors and/or alter the actions of EPO. While the mechanisms of action are important, these studies bring up another key issue, which is that the actions of a single cytokine in a controlled, cell culture model are unlikely to represent the actions of the

cytokine *in vivo*. The co-release of other cytokines, the microenvironment in question (spleen versus circulation, for example), and the model used are all likely to alter the results and is likely why the field has seemingly contradictory results. Importantly, there are also some cytokines that may inhibit part of the erythropoiesis pathway but stimulate another. IL-6 is one example in which it can stimulate EPO production in the presence of hypoxia but can inhibit erythroid precursor cell proliferation. Its ultimate role in modulating Hb mass in an intact human model is largely unknown. Therefore, while not in its infancy of exploration, the topic of immune regulation of erythropoiesis *in vivo* is still incompletely understood.

Immune system and pulmonary pressure regulation

It has been shown that SCUBA diving alters endothelial function (Nossum *et al.*, 1999, 2002; Brubakk *et al.*, 2005; Madden *et al.*, 2010; Culic *et al.*, 2014) and immune system activity, as discussed above (Marabotti *et al.*, 1999; Nossum *et al.*, 2002; Thom *et al.*, 2013; Eftedal *et al.*, 2013; Sureda *et al.*, 2014; Madden *et al.*, 2014; Žarak *et al.*, 2021; Rocco *et al.*, 2021). Therefore, baseline inflammatory cytokine expression and/or the change in cytokine expression after a dive may play a regulatory role in the changes in pulmonary pressure with SCUBA diving.

There is ample evidence that inflammatory cytokines can regulate pulmonary vascular tone (Groth *et al.*, 2014). However, important to this discussion is the context in which each cytokine regulates pulmonary vascular tone. Most studies examine these cytokines in the context of pulmonary artery hypertension (PAH), of which there are multiple types. Most literature in the clinical field focuses on the association of elevated cytokines with PAH, and in some cases, tests the efficacy of anti-inflammatory treatments on reducing symptoms of PAH (Groth *et al.*, 2014). Rarely do these studies focus on mechanisms by which inflammatory cytokines may act on

pulmonary vascular smooth muscle cells. In addition, PAH is a chronic condition and is not defined by a sustained pulmonary vasoconstriction but rather by pulmonary vascular remodeling (Fujita *et al.*, 2002). Some cytokines can act on pulmonary vascular smooth muscle cells but only in a way that promotes cellular proliferation or some additional aspect of vascular remodeling and not vasoconstriction itself (Groth *et al.*, 2014). Conversely, we are examining acute elevations in pulmonary pressure only. This section of the literature review will focus on those few mechanistic studies to show that inflammatory cytokines can directly act on pulmonary vascular smooth muscle cells and therefore may promote vasoconstriction. Importantly, no studies to date have examined inflammatory cytokine regulation of pulmonary vascular tone in SCUBA diving, so understanding the mechanisms by which these cytokines can directly act on the pulmonary vasculature independent of SCUBA diving is important in framing **Aim 3**.

In isolated rat pulmonary arteries, IL-1β and TNF-α have a regulatory role in HPV (Tsai *et al.*, 2004). While we are not directly studying HPV, these data emphasize the role of cytokines in acting directly on the pulmonary vasculature acutely. These authors reported a biphasic HPV response, similar to those studies in humans (Talbot *et al.*, 2005; Sylvester *et al.*, 2012; Swenson, 2013). Importantly, IL-1β and TNF-α expression were significantly increased with hypoxia specifically in the pulmonary artery tissue (Tsai *et al.*, 2004), and since the pulmonary artery ring preparations included intact endothelium, it is likely that the pulmonary artery endothelial cells were producing the cytokines. In addition, Tsai *et al.* (2004) examined the pathway through which these cytokines might cause vasoconstriction and found it to be protein kinase C (PKC) dependent. Blocking PKC reduced the expression of IL-1β and TNF-α and subsequently the strength of the phase II HPV, and activating PKC resulted in the opposite effect (Tsai *et al.*,

2004). Therefore, it is likely that the role of PKC in regulating HPV is dependent on increasing IL-1β and TNF-α expression.

There is no evidence for the role of interferons (IFN-α and IFN-γ) on HPV, but there is evidence for their role in PAH and the mechanism by which they alter PAH. These data should be interpreted cautiously since this dissertation does not aim to study pathophysiological chronic elevations in pulmonary pressure. Rather, these data are provided to show a potential regulatory role that interferons have on the pulmonary vasculature that is independent of vascular remodeling that occurs with PAH. Interestingly, the role of interferons in regulating pulmonary vascular tone came to be understood accidentally, as PAH was found to be a rare side effect of interferon therapy (Kramers *et al.*, 1993; Savale *et al.*, 2016; Papani *et al.*, 2017). In one study, it was found that both IFN-α and IFN-γ induced the expression of endothelin-1, a potent vasoconstrictor, in pulmonary artery smooth muscle cells but not endothelial cells (George *et al.*, 2014). Importantly, the pulmonary artery smooth muscle cells were primed with TNF-α, so it may only be a combined effect of the interferons with TNF-α that result in an increased endothelin-1 expression (George *et al.*, 2014).

IL-6 has been studied extensively in the context of PAH (Groth *et al.*, 2014). However, little research exists to its role acutely in pulmonary vascular tone regulation. A study by Savale *et al.* (2009) examined the effect of IL-6 on the acute and chronic pulmonary pressure responses to hypoxia. They found that acutely, wild type and IL-6 knockout mice had the same increase in right ventricular systolic pressure in response to hypoxia (Savale *et al.*, 2009). Chronically, IL-6 knockout mice showed lower right ventricular hypertrophy and less muscularization of the pulmonary artery, indicating that IL-6 likely affects pulmonary remodeling rather than acute responses to hypoxia (Savale *et al.*, 2009).

There are no data to suggest that the chemokines studied in this dissertation, IL-8 and MCP-1, are involved in acute regulation of pulmonary vascular tone. However, data exists to support MCP-1's role in PAH progression. A study by Kimura *et al.* (2001) showed that IL-1β, TNF-α, and MCP-1 were all elevated in PAH patients but only MCP-1 positively correlated with pulmonary vascular resistance. IL-8 has similarly been implicated in PAH but not acute elevations in pulmonary pressure (Scott *et al.*, 2019).

IL-10, IL-18, IL-23, and IL-33 have no known role in acute elevations in pulmonary pressure. IL-33 has been shown to cause vascular remodeling in pulmonary hypertension by acting on vascular endothelial cells (Liu *et al.*, 2018*a*), but this cytokine has yet to be shown to play an acute role in pulmonary pressure regulation. Alternatively, given its similarities to IL-1, IL-18 is similarly implicated in PAH (Groth *et al.*, 2014), with elevated concentrations of IL-18 seen in pulmonary vascular smooth muscle cells (Ross *et al.*, 2012). IL-1β also regulates HPV (Tsai *et al.*, 2004), so perhaps IL-18 also plays a similar, albeit unknown, regulatory role. IL-10 administration has been shown to reduce mean pulmonary artery pressure in patients with PAH (Ito *et al.*, 2007), but no acute mechanisms of action have been tested. Lastly, IL-23 has no known role in pulmonary pressure regulation acutely or in PAH.

Preliminary data from our laboratory suggest that baseline concentrations of IL-17A positively correlate with 4- and 7-hour PASP and TPR in response to 11.5% oxygen, indicating an acute role of this cytokine in pulmonary pressure regulation with hypoxia. In addition, further support for the role of IL-17 comes from a study by Wang *et al.* (2019*b*) in which they found elevated IL-17 in lung tissue of humans with COPD-associated pulmonary hypertension as well as mice with hypoxic pulmonary hypertension. In a series of mechanistic studies, it was shown that IL-17 contributes to hypoxic pulmonary hypertension by inducing proliferation,

angiogenesis, and adhesion of pulmonary artery endothelial cells (Wang *et al.*, 2019*b*), and blocking IL-17 prevented these actions. However, this work required 4 weeks of hypoxia, so it was not an acute model. Thus, although our data suggest an acute role of this cytokine with hypoxia-induced increases in pulmonary pressure, no direct acute mechanistic studies exist. In addition to IL-17, data from our laboratory show that baseline concentrations of IL-12p70 positively correlate with 7-hour pulmonary PASP and TPR in response to 11.5% oxygen. No additional studies exist to show the role of IL-12 acutely on pulmonary pressure regulation, but IL-12 expression is higher in patients with PAH (Soon *et al.*, 2010; Rabinovitch *et al.*, 2014).

Importantly, no studies have investigated the regulatory role of cytokines on pulmonary pressure in either hyperoxia or hyperbaria. Therefore, an important question to answer is whether the relationships identified and discussed above between inflammation and pulmonary vascular tone regulation also exist after SCUBA diving. The data support a role of IL-1β and TNF-α in regulating acute increases in pulmonary pressure with hypoxia, but limited data exists as to whether the other cytokines in question will play a role in the post-SCUBA diving induced increase in pulmonary pressure. Taken together, these studies emphasize that the role of the immune system on acute regulation of pulmonary vascular tone is incompletely understood, and no studies have examined a modulatory role of the immune system on pulmonary pressure regulation post-SCUBA diving.

IRON REGULATION

Essential to red blood cell formation is the bioavailability of iron. As noted above, the Hb molecule contains various heme groups that bind oxygen. Therefore, Hb cannot be formed without dietary iron. Iron deficiency is the leading cause of anemia, particularly in females (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; Cappellini *et al.*, 2020),

and presents a serious pathological condition by limiting oxygen delivery to tissues. In addition to total iron, iron availability can be regulated by the state of the iron. In every person, a small portion of the total Hb has its iron atoms in the inactive ferric state, called methemoglobin. Furthermore, when discussing iron status, it is important to consider ferritin and transferrin concentrations as well as total iron. Ferritin is the stored form of iron, and transferrin is necessary for the transport of iron once ferritin is mobilized. Therefore, low ferritin and high transferrin may be indicative of reduced iron availability even in the face of normal total iron concentrations. Lastly, hepcidin is a protein that is the main regulator of iron metabolism. It is made in the liver and functions to sequester iron and prevent the mobilization of iron stores. Hepcidin also inhibits the release of recycled iron from macrophages (Ganz, 2007, 2016).

Although its most well-known function is increasing EPO transcription, HIF-2 has also been shown to help with iron uptake and utilization (Haase, 2013). During hypoxia, HIF-2 stabilization will increase bioavailability of iron in order to make more red blood cells (Haase, 2013). In addition, erythropoiesis will inhibit hepcidin to increase iron availability for erythropoiesis (Ganz, 2007). However, intracellular iron can provide negative feedback on this process, as low intracellular iron concentrations were found to suppress HIF-2α translation (Haase, 2013), subsequently reducing EPO production. Therefore, in humans who have low iron, EPO hyporesponsiveness to hypoxia may be partially explained by low iron availability, which can be determined by examining complete iron status rather than iron alone.

The immune system can also regulate iron availability. During infection, iron is sequestered into macrophages and hepatocytes, iron uptake is limited, and expression of iron storage proteins like ferritin are increased to limit iron availability to pathogens (Slaats *et al.*, 2016; Ni *et al.*, 2022). In addition, many of the cytokines of interest for this dissertation can

regulate iron availability. For example, much of the work on how IL-6 regulates erythropoiesis shows that it modulates Hb mass through its regulation of the protein hepcidin. IL-6 can increase hepcidin expression (Nemeth *et al.*, 2004; Banzet *et al.*, 2012), which reduces iron availability for red blood cell synthesis. Specifically, IL-6 is known to increase hepcidin through the induction of STAT3, which binds to the promoter region of the hepcidin gene to increase its transcription (Nemeth *et al.*, 2004; Wrighting & Andrews, 2006; Wessling-Resnick, 2010; Banzet *et al.*, 2012; Fraenkel, 2017). Conversely, IL-10 treatment can alleviate anemia of inflammation by reducing inflammatory cytokine expression and downregulating hepcidin (Huang *et al.*, 2017). In fact, excessive IL-10 was shown to contribute to high altitude polycythemia (Liu *et al.*, 2018*b*). IL-10 acts through the STAT3 pathway to directly oppose IL-6 (Huang *et al.*, 2017). MCP-1 targets iron via a different pathway in which it suppresses the transferrin receptor (Muckenthaler *et al.*, 2011). In mice with MCP-1 knockouts, there was decreased blood iron and transferrin saturation as well as iron overload in the spleen and duodenum (Muckenthaler *et al.*, 2011). IFN-γ decreases iron availability to pathogens by promoting iron export from macrophages (Abreu *et al.*, 2020), which may increase circulating iron. Alternatively, TNF-α induces hypoferremia by inhibiting macrophages from releasing iron and increasing iron storage (Alvarez-Hernández *et al.*, 1989; Scaccabarozzi *et al.*, 2000), and in endothelial cells, TNF-α has been found to induce iron accumulation (Nanami *et al.*, 2005). As noted above, IL-33 is necessary for the development of red pulp macrophages (Lu *et al.*, 2020; Xu & Huang, 2020). These macrophages recycle iron in red blood cells, and in IL-33 deficient mice, iron recycling was impaired and there was increased iron deposition in the spleen (Lu *et al.*, 2020). Thus, in summary, these studies suggest that high concentrations of inflammatory cytokines may limit iron availability for red blood cell synthesis.

Iron availability can also influence expression of cytokines and immune cells. In iron overloaded myelomonocytic cells, IL-1β production in response to LPS administration was reduced, and upon iron chelation, IL-1β production was augmented (O'Brien-Ladner *et al.*, 2000). Additionally, iron overload is associated with increased susceptibility to many disease states, such as tuberculosis and malaria, whereas iron deficiency is associated with increased resistance to infection (Wessling-Resnick, 2010). Iron acts on nearly all immune cells, including macrophages, NK cells, neutrophils, and T- and B-lymphocytes (Ni *et al.*, 2022). Iron regulates macrophage polarization into various subtypes of macrophages, regulates neutrophil recruitment and neutrophil derived inflammation, and inhibits T cell differentiation into Th1, Th2, and Th17 subtypes (Ni *et al.*, 2022). Together, these data support a role of iron and immune interactions as a regulatory component of Hb mass as well as EPO concentrations in response to hypoxia.

PATENT FORAMEN OVALE

Normal fetal circulation is structured differently than that of a developed adult human. Since the fetus is not breathing on its own and receives oxygen from the mother through gas diffusion across the placenta, there are pathways in the fetal circulatory system that allow blood to bypass the lungs. These include the ductus arteriosus, a vessel connecting the aortic arch to the pulmonary artery, and the foramen ovale, a tunnel connecting the right and left atria. In utero, the fetus is hypoxic, which promotes higher right atrial pressures, but not left atrial pressures (Malik & Kidd, 1976; Groves *et al.*, 1987), so the pressure gradient largely drives blood flow from the right to the left atria through the foramen ovale, where it can then enter systemic circulation from the left side of the heart.

When an infant takes its first breaths, the pressure gradient across the atria changes. Given the relatively hyperoxic environment for the newborn, the pressure in the right atrium

drops, and left atrial pressure largely exceeds that of the right atria due to reductions in pulmonary vascular resistance and increased pulmonary blood flow. This favors near immediate functional closure of the foramen ovale. After a few months, the cells around the foramen ovale undergo endothelial-to-mesenchymal transition leading to fibrosis, and anatomical closure occurs (Elliott *et al.*, 2014*a*). However, in some cases, for reasons currently unknown, the foramen ovale does not close, and it is termed a PFO.

A PFO is a remnant fetal circulatory system structure present in approximately 25-40% of the adult population (Hagen *et al.*, 1984; Marriott *et al.*, 2013; Elliott *et al.*, 2013). It is an intracardiac right-to-left shunt. Accordingly, it would not be unexpected to hypothesize that a PFO might present a mechanism of increased arterial hypoxemia. Arterial hypoxemia could result in decreased oxygen delivery to the kidney, resulting in a compensatory erythropoiesis and higher baseline Hb mass. It could also result in increased pulmonary vasoconstriction and therefore higher pulmonary pressures. In support of this, in a study by Lovering *et al.* (2011), participants with a PFO (PFO+) had lower PaO_2 of approximately 5mmHg at rest and had worse resting gas exchange efficiency. However, the PaO_2 in those participants with a PFO was still on average 94mmHg, which does not represent a large degree of arterial hypoxemia (Lovering *et al.*, 2011). Additionally, Duke *et al.* (2020) found no differences between those with and without a PFO in arterial hypoxemia at sea level even though PFO+ participants had worse gas exchange efficiency. An important consideration for whether a PFO causes arterial hypoxemia are sex and PFO size. Preliminary data from our lab suggests that females with a large PFO have worse gas exchange efficiency and arterial hypoxemia at rest but that males with a PFO do not have worse gas exchange efficiency or arterial hypoxemia (Kelly *et al.,* 2023. In Review). It could be that those with a small PFO, those with normal right and left heart pressures, and most males do not

exhibit a significant amount of shunting at rest and therefore do not have worse pulmonary gas exchange efficiency and/or arterial hypoxemia.

Despite studies suggesting those with a PFO would have higher pulmonary pressure in response to hypoxia compared to those without a PFO due to increased PFO driven susceptibility to high altitude pulmonary edema (Allemann *et al.*, 2006), there were no differences in pulmonary pressures between those with and without a PFO after 30 minutes of varying degrees of hypoxia (Duke *et al.*, 2020), and in this same set of participants, there were no PFO driven differences in PASP or TPR with 10 hours of normobaric hypoxia (DiMarco *et al.*, 2021). However, as noted above, Duke *et al.* (2020) found no arterial hypoxemia in those with a PFO at rest, so it could be that only those that shunt more at rest may have a greater stimulus PO_2 needed to observe greater HPV.

Accordingly, simply the presence of a PFO is not expected to result in differences in Hb mass (**Aim 2**) or pulmonary pressure with SCUBA diving (**Aim 3**) given that 1) there is likely not a significant degree of arterial hypoxemia without considering sex or PFO size, and 2) no studies have shown that those with a PFO have higher pulmonary pressures under normoxic or hypoxic resting conditions. However, some considerations that have yet to be studied are differences in immune system activity and iron regulation in those with a PFO. There is data to suggest a link between the presence of a PFO, iron deficiency, and stroke risk, although this link has not been confirmed (Shovlin, 2014; Topiwala *et al.*, 2021). Therefore, if the presence of a PFO does result in some degree of iron deficiency, this may negatively regulate Hb mass. There is no data to suggest that the presence of a PFO alters inflammation, but if the presence of a PFO alters inflammation, it has the potential to alter Hb mass and/or pulmonary pressure.

CHAPTER III

METHODS

INFORMED CONSENT

All protocols completed for this dissertation were approved by the University of Oregon Research Compliance Services (protocol #s STUDY00000174, STUDY00000189, STUDY00000019, and 07302018.031). Additionally, the University of Split School of Medicine Ethics Committee approved all research activities for **Aim 3** (protocol #2181-198-03-04-19-0052), and **Aim 3** is registered at clinicaltrials.gov (protocol #07302018.031). I verbally discussed all procedures, risks, and benefits with each participant, and each participant completed written informed consent prior to participation in any research activities. For **Aim 3** (Chapter VI), many participants spoke Croatian as a first language, so a researcher fluent in Croatian (Otto Barak, MD) was able to aid in the informed consent process and appropriately answer any questions the participants might have.

HB MASS & COi

The gold standard for measuring Hb mass is the CO rebreathe technique (Siebenmann *et al.*, 2017). This technique was used in **Aims 1** and **2** (Chapters IV and V, respectively). For this test, participants breathed on a custom-made Hb mass circuit. The circuit consisted of a one-way open circuit, which was connected to a Douglas bag, and a closed-circuit, which was used for the CO rebreathing. A sliding valve (Series 2870, Hans Rudolph, Shawnee, KS, USA) was used to switch between the open and closed circuits. To administer CO into the closed circuit, a 200mL syringe was mounted to a custom metal plate with a metal stopper that could be positioned at increments of 10mL. A small 3-way stopcock was attached to the end of the CO syringe, which was used to prevent contact with room air as well as attach to the closed circuit for CO

administration. Figure 3 diagrams the setup of the complete Hb mass circuit. Prior to any testing, the deadspace in the circuit was directly measured with water and remained constant throughout all studies.

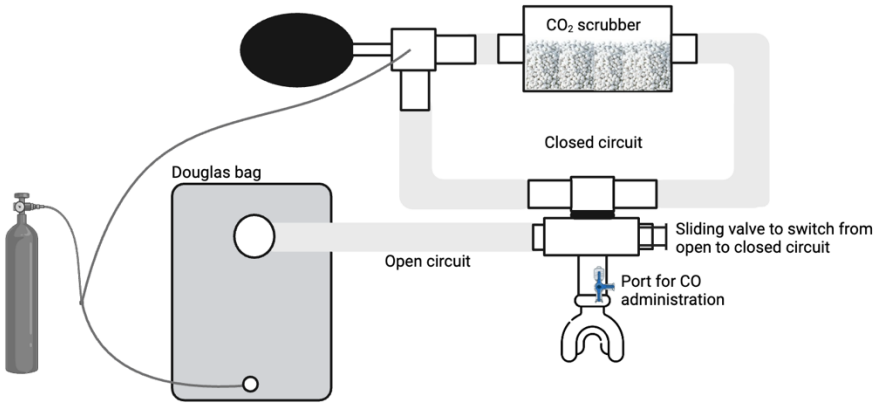


Figure 3. Hb mass rebreathe circuit. The circuit consisted of two connected circuits. One was an open circuit used to breathe 100% oxygen from a Douglas bag and exhale into the room, and the other was a closed circuit used to rebreathe a bolus of CO in 100% oxygen for 10 minutes. A sliding valve connected to one-way non-rebreathe valves was used to switch between the two circuits. A T-shaped connector was used to connect a piece of tubing to the Douglas bag to fill the closed circuit with oxygen as needed as well as connect a rubber balloon to the circuit to allow the participant to breathe. The gas tank pictured contains 100% oxygen.

Upon arrival, participants began by having height and weight measured. Weight was used to calculate the volume of CO to administer during the CO rebreathe (0.8 mL of CO/kg of body mass for females and 1.0 mL of CO/kg of body mass for males rounded to the nearest 10mL). This volume of CO was chosen to raise HbCO by approximately 4-7% in a single test. All Hb mass tests were performed in duplicate on the same day, so HbCO was raised by approximately

8-14% by the end of the duplicate measures. After having weight measured, participants had an intravenous catheter (IV) placed in an antecubital vein (See "*IV placement & blood draw procedure*" below). The IV was used for various small blood draws to measure HbCO. Room temperature and barometric pressure were recorded. After instrumentation, the participant began the Hb mass test.

To begin the test, the participant laid down in the supine position with their feet elevated approximately 8 inches with a small box that they could prop their legs on. The participant remained laying down for 20 minutes for the normalization of plasma volume shifts due to gravity (Hagan *et al.*, 1978; Keiser *et al.*, 2013). During this time, the Douglas bag connected to the one-way open circuit was filled with 100% oxygen. Additionally, the closed circuit was filled and flushed three times with 100% oxygen. After 20 minutes of supine rest, the participant began breathing on the mouthpiece connected to the open circuit, where they breathed 100% oxygen for 4 minutes (hyperoxia period). The purpose of the four minutes of hyperoxia was to lower baseline HbCO and increase SaO_2 by as much as possible. During the 4-minute hyperoxia period, the CO syringe was filled and flushed 3 times with the pre-calculated volume of CO based on body weight, then filled to the desired volume. To avoid over-pressurizing the syringe and therefore administering too much CO, a small piece of tubing was connected to the 3-way stopcock at the end of the syringe. The end of the tubing was placed into a small water column (5cm H_2O). The 3-way stopcock was then opened between the CO syringe and tubing to allow equalization of pressures and then promptly closed. At the end of the hyperoxia period, a small venous blood sample was drawn from the IV into a pre-heparinized syringe. After, the participant was instructed to exhale completely then switched to breathe on the closed circuit, and the CO was immediately injected into the closed circuit. Upon injecting all the CO, a timer

began for 10 minutes of CO rebreathe. At the end of 10 minutes, another small blood sample was drawn as before. The participant was again instructed to exhale completely, the closed circuit was shut, and the participant came off the mouthpiece and remained laying in the supine position breathing room air for 10 minutes.

While the participant was resting between measures, the volume of air and the parts per million (ppm) of CO remaining in the rebreathe circuit were measured. The volume was measured by attaching a large 3-way stopcock to the circuit and using two 3-liter calibration syringes to draw out the remaining gas. The volume was measured to the nearest 1/10 of a liter. The air was then injected back into the closed circuit. After, a CO monitor (CO-220 Carbon Monoxide Meter, Fluke, Everett, WA) was held to the opening of the circuit where the mouthpiece attached. The air in the circuit was slowly squeezed out, and the maximum reading on the CO monitor was recorded.

Following the volume and ppm measurements, both open and closed circuits were refilled and flushed with 100% oxygen, and the participant then completed the 4-minute hyperoxia period followed by 10-minute CO rebreathe once again as described above. All 4 blood samples collected were analyzed via co-oximetry to obtain HbCO before and after CO rebreathing for each of the duplicate measures (See '*co-oximetry'* below). To calculate Hb mass, the volume of CO administered, change in HbCO, atmospheric temperature and pressure, participant height and age, deadspace of the circuit, volume of gas remaining in the circuit, and ppm of CO remaining in the circuit were used. Participant age and height were used to estimate residual volume, which was used with the deadspace of the circuit, volume of gas remaining in the circuit, and ppm of CO remaining in the circuit to calculate the volume of CO that was not taken up or absorbed by the participant. The volume of CO unabsorbed was subtracted from the

volume of CO administered to obtain the volume of CO absorbed by the participant. Using atmospheric temperature and pressure, the volume of CO absorbed was converted into moles, and then converted into moles of total Hb using the change in HbCO. Moles was then converted into grams to achieve the total grams of Hb. Percent error was calculated as the standard deviation of the duplicate measures divided by the square root of 2, which was all divided by the mean of the duplicate measures, multiplied by 100 to express as a percent. The test was considered valid if the percent error between duplicate measures was less than 3%. A sample calculation of Hb mass and the percent error are provided in the *Calculation appendix.* Hb mass was measured for **Aim 2** (Chapter V) as a main outcome variable, but this protocol was also employed to administer CO to participants for **Aim 1** (Chapter IV).

HOT WATER IMMERSION

Heat stress for **Aim 1** (Chapter IV) was done via HWI. All participants arrived between 06:30 and 08:30 and were asked to arrive well hydrated. Hydration was assessed with urine specific gravity upon arrival, and if a participant had urine specific gravity below 1.020, they were asked to consume 5 mL/kg of body weight of water prior to starting HWI.

The hot tub was heated to 40°C (104°F). While in the hot tub, participants were seated in an upright position submerged to approximately the level of the shoulders. The participant was instrumented with an IV catheter in their right arm and blood pressure cuff on their left arm, so both arms remained unsubmerged during HWI. All participants had body core temperature monitored via either a rectal thermistor or a telemetric core temperature pill. If participants opted to use a core temperature pill (HQInc CorTemp Sensor, Palmetto, FL), they came to the lab the day prior to their study visit and were instructed to ingest the pill approximately 10 hours prior to their scheduled arrival time (approximately 20:30-22:30). If a participant opted to use the rectal

thermistor probe (ZOLL Medical Corporation YSI Reusable rectal temperature probe, Chelmsford, MA) they were instructed on how to self-insert the thermistor and provided with lubricant. While in the hot tub, we sought to increase core temperature to 38.5°C with water immersion up to the sternum. In the event that core temperature of 38.5°C was reached, water immersion up to the waist was performed and a fan turned on in an attempt to clamp core temperature at 38.5°C to improve participants' thermal perception at this elevated core temperature.

SCUBA DIVING

For **Aim 3** (Chapter VI) participants all completed a recreational, non-decompression SCUBA dive. All participants held at least their open water SCUBA dive certification through the Professional Association of Diving Instructors (PADI) or their 1 Star certification through Confédération Mondiale des Activités Subaquatiques (CMAS) and were recruited from professional and local dive clubs and organizations in and around Split, Croatia. Participants were instructed to maintain either an 18-meter dive profile for 47 minutes of bottom time or a 30-meter dive profile for 20 minutes of bottom time. Both times were chosen as the longest a person can spend at those respective depths without requiring decompression on the ascent. The shallower dive profile was considered moderately deep and was chosen to allow participants with a range of experience to be able to complete the dive with one tank of gas, while the deeper dive profile is deeper than an Open Water Certified Diver is allowed to dive and required an Advanced Open Water certification. Participants all wore dive computers (Scubapro Mantis 2, El Cajon, CA) that continuously monitored dive depth, dive time, and water temperature. All participants dove with at least one dive buddy, and there were no complications because of the dive profiles. Each participant completed two dives for the study on back-to-back days, with the

exception of one participant who completed his dives two days apart. Three participants completed both dive profiles.

DRUG TREATMENT

For **Aim 3** (Chapter VI), participants received sildenafil and placebo in a balanced, randomized crossover design for females and males. Sildenafil was given in an oral dose of 50mg, which has been shown to be effective at reducing hypoxic pulmonary vasoconstriction (Carter *et al.*, 2019; Kelly *et al.*, 2022) and pulmonary artery and pulmonary wedge pressure in those susceptible to immersion pulmonary edema (Moon *et al.*, 2016; Martina *et al.*, 2017) but is a small enough dose to minimize unwanted side effects such as headache. Half of the participants received sildenafil after their first dive, and half of the participants received sildenafil after their second dive. The ultrasonographer, participants, and investigators making measurements and calculations were blinded to the treatments.

Upon resurfacing from the dive, participants were given their assigned drug treatment for the day. The first participant ingested their assigned drug treatment immediately upon resurfacing, and every 15 minutes thereafter the next participant ingested their assigned drug. Post-dive measurements were made one hour after ingesting the drug, which is the time it takes sildenafil to reach its peak effect.

ULTRASOUND MEASURES

Comprehensive ultrasound

Comprehensive cardiac ultrasound was performed as part of the screening procedures for all participants in **Aim 3** (Chapter VI) of this dissertation. All comprehensive cardiac ultrasound was done by a licensed registered diagnostic cardiac sonographer (Joel Eben Futral, Freddy Garcia, Doug Elton, and Dr. Rachel Lord) due to the technical difficulty of obtaining accurate

images of the heart. The purpose of the comprehensive cardiac ultrasound was to screen out participants with potential cardiac limitations that might have prevented their safe participation in the study; however, no participant was screened out of the study due to a finding during the comprehensive ultrasound. All measured were done according to standards set by the American Society of Echocardiography (Lang *et al.*, 2015; Mitchell *et al.*, 2019).

Participants all had a 3-lead electrocardiogram placed and were positioned in a reclining phlebotomy chair. Measures obtained by the comprehensive echocardiogram included right ventricular wall thickness, interventricular septum thickness at end diastole, left ventricular internal dimension at end diastole, left ventricular internal dimension at end systole, left ventricular posterior wall thickness at end diastole, left atrial size, pulmonic valve peak velocity, left ventricular outflow tract velocity, aortic valve velocity, aortic valve velocity time integral, aortic valve area, mitral valve ejection velocity, mitral valve acceleration velocity, early diastole velocity waveform, ratio of E wave to e' wave, right ventricular end diastolic area, right ventricular end systolic area, ventricular ejection fraction, left ventricular end diastolic volume, left ventricular end systolic volume, heart rate at the left ventricular volume measurement, left ventricular ejection fraction, diameter of the inferior vena cava (IVC), and IVC collapsibility.

IVC diameter and collapsibility were used to estimate right atrial pressure (P_{RA}). IVC collapsibility was calculated as the percentage difference between the maximal and minimal IVC size upon a rapid sniff. If the IVC diameter was less than 2.1cm and the IVC had greater than 50% collapsibility, P_{RA} was assigned a value of 3mmHg. If the IVC diameter was greater than 2.1cm and the IVC had greater than 50% collapsibility, P_{RA} was assigned a value of 8mmHg. If the IVC diameter was less than 2.1cm and the IVC had less than 50% collapsibility, P_{RA} was

assigned a value of 8mmHg. Lastly, if the IVC diameter was greater than 2.1cm and the IVC had less than 50% collapsibility, P_{RA} was assigned a value of 15mmHg (Bamira & Picard, 2018).

PFO screening

The presence of a PFO can be detected via direct catheterization, transesophageal echocardiography, or transthoracic echocardiography. Because transthoracic echocardiography is the least invasive and most accessible to research participants, our lab uses this method for the detection of a PFO. All PFO screening was done by a licensed registered diagnostic cardiac sonographer (Joel Eben Futral, Freddy Garcia, Doug Elton, and Dr. Rachel Lord) due to the technical difficulty of obtaining accurate images of the heart.

PFO screening was done with a procedure called transthoracic saline contrast echocardiography, which has been validated to detect the presence of a PFO and estimate the severity of the shunt (estimate of PFO size) against direct invasive measures (Fenster *et al.*, 2014). When participants arrived, they had an IV catheter placed into a peripheral vein in the upper limb (See "*IV placement & blood draw procedure*" below). The participant lied semi-recumbent in a phlebotomy chair in the left lateral decubitus position. This position allowed the heart to be closest to the ribcage for easier imaging. The sonographer placed a small probe against the left ribcage of the participant near the apex of the heart. This allows the sonographer to visualize all 4 chambers of the heart, and the image they obtain is called the 4-chamber apical view. Cardiac tissue is visualized as white, and blood in the heart chambers is visualized as black. Once the sonographer obtained a good view of the heart, two 10mL syringes were attached to the 3-way stopcock connected to the participant's IV. Saline and air were agitated between the two syringes. Saline agitation creates microbubbles which were then rapidly injected into the IV. The ultrasound was used to visualize the bubbles, which appeared opaque. Upon

injection of the microbubbles, the bolus of bubbles appeared on the right side of the heart. If the participant had a PFO, some bubbles appeared on the left side of the heart within three heartbeats.

Blood flow through a PFO is dependent on right atrial pressure exceeding left atrial pressure. Therefore, to visualize a PFO, the microbubbles must be injected and coincide with a time when right atrial pressure exceeds left atrial pressure. This occurs naturally when the end of an inspiration coincides with end of ventricular diastole and start of ventricular systole (Strunk *et al.*, 1987; Fenster *et al.*, 2014). However, during most resting, sea level conditions right atrial pressure does not exceed left atrial pressure. Rather than try to time the injection of microbubbles so that they appear in the right side of the heart during end inspiration and diastole to allow conditions in which blood will flow through a PFO, we had participants perform a Valsalva maneuver. To perform this maneuver, participants bore down using their abdominal muscles, similar to bearing down during a bowel movement. This allowed for a transient increase in intrathoracic pressure, which inhibited blood flow returning to the right side of the heart. Upon release of the Valsalva, the surge in blood flow returning to the right atrium created a situation in which right atrial pressure exceeded left atrial pressure and blood readily flowed through a PFO if present.

A participant was said to have a PFO (PFO+) if any number of microbubbles appeared on the left side of the heart within three heartbeats. Furthermore, the sonographer graded the PFO on a scale of 1-5 depending on the number of bubbles seen in the left atrium. Grades 1-2 had fewer than 12 bubbles seen in the left atrium. Grade 3 indicated greater than 12 bubbles appearing in the left atrium. Grade 4 represented an uncountable number of bubbles that appeared heterogeneously in the left atrium, and grade 5 indicated an uncountable number of

bubbles that appeared homogenously in the left atrium. The bubble scoring system is designed to approximate the size of the PFO, with grades 1-2 representing a small PFO and grades 3-5 representing a large PFO. However, measuring the true size of the PFO or the amount of blood traveling through the PFO is beyond the scope of this dissertation, as it requires more direct measures. Therefore, while the terms 'large PFO' and 'small PFO' may be used, the interpretation of these terms as the true size of the PFO should be done with caution.

A participant was said to have no PFO (PFO-) if no bubbles appeared in the left atrium. However, some participants had late bubbles, which were defined as bubbles appearing in the left atrium later than three heartbeats. These late bubbles were attributed to large diameter intrapulmonary shunt pathways that allowed for the bubbles to pass through pulmonary circulation without being filtered out in the small capillaries. Pulmonary transit time of blood is estimated to be about 9.3 seconds at rest (Hopkins *et al.*, 1996). Therefore, in a person with a resting heart rate of 60 bpm, it would take approximately 9 cardiac cycles for blood to travel through pulmonary circulation and through any potential intrapulmonary shunts. Additionally, assuming a normal stroke volume of 70mL per beat and a pulmonary blood volume of 210mL, it would take 4 heart beats for blood pumped out of the right ventricle to return to the left atrium. Saline contrast would not appear on the left side of the heart within 3 heart beats if a participant had intrapulmonary shunts but no PFO, so any participant with late bubbles were considered to have no PFO.

PFO screening was performed for **Aims 2** and **3** (Chapters V and VI, respectively) of this dissertation. **Aim 2** (Chapter V) combined data collected from three studies (study 1, study 2, and study 3). Those that participated in studies 2 and 3 were imaged for the presence of absence of a PFO, while those that participated in study 1 were not.

Right heart measures

For **Aim 3,** various right heart measures (right ventricular outflow tract (RVOT) velocity time integral (VTI), RVOT acceleration time (RVOT AT), RVOT ejection time (RVOT ET), right ventricular end diastolic (RVED) area, right ventricular end systolic (RVES) area, right ventricular diameter at the base (RV dim base) and middle (RV dim mid), and tricuspid annular plane systolic excursion (TAPSE)) were taken pre- and post-SCUBA diving to confirm normal right heart function (Lang *et al.*, 2015). This measurement was also done to examine whether changes in pulmonary pressure and resistance were associated with changes in right heart function. All measurements were made in triplicate and triplicate values averaged.

Pulmonary artery systolic pressure, cardiac output, and total pulmonary resistance

The following measurements were performed for **Aim 3** (Chapter VI) of this dissertation. PASP was measured to represent the peak pressure exerted upon the pulmonary arteries during right ventricular systole. This can be estimated by measuring the peak velocity of retrograde blood flow across the tricuspid valve (*v*). During systole when the right ventricle contracts, the tricuspid valve closes, but a small amount of blood can still flow backwards into the right atrium. The peak retrograde blood flow across the tricuspid valve can therefore represent the pressure gradients across the right ventricle and atrium. Because right ventricular pressure can change with pulmonary artery pressure, a larger *v* is indicative of higher PASP. The method used in this dissertation is an indirect measure of pulmonary pressure. Direct right heart catheterization can provide more direct measurements and allows for measurement of mean pulmonary artery pressure rather than PASP. However, studies have shown that indirect doppler ultrasound correlates strongly with measures obtained during direct heart catheterization (Yock & Popp, 1984; Himelman *et al.*, 1989*a*).

v was measured pre- and post-SCUBA diving in triplicate, and triplicate values were averaged. The average *v* was then applied to the modified Bernoulli equation (*equation 3, "Equations appendix"*), as we and others have previously done (Yock & Popp, 1984; Himelman *et al.*, 1989*b*; Rudski *et al.*, 2010; Laurie *et al.*, 2010, 2012; Norris *et al.*, 2014*a*; Duke *et al.*, 2014; Elliott *et al.*, 2014*b*; Duke *et al.*, 2016, 2020; DiMarco *et al.*, 2021). PASP is calculated as 4 times the *v* squared plus the P_{RA} (*equation 3, "Equations appendix"*). P_{RA} pressure is estimated from the IVC diameter and collapsibility as described above in "*comprehensive ultrasound*".

Q was calculated by measuring the left ventricular outflow tract (LVOT) VTI and diameter. LVOT diameter was used to calculate the cross-sectional area of the LVOT. The LVOT diameter was divided by two to obtain the radius of the LVOT, which was then squared and multiplied by pi (approximated 3.14) (*equation 4, "Equations appendix")*. LVOT VTI was measured pre- and post-SCUBA diving in triplicate, and the triplicate values were averaged. The average LVOT VTI was multiplied by the cross-sectional area of the LVOT to obtain an estimate of stroke volume. The heart rate measured with a 3-lead ECG was multiplied by stroke volume to obtain Q.

TPR was calculated as the PASP divided by Q. however, while vascular resistance is primarily a function of the vessel radius, blood viscosity is an additional variable that should be taken into consideration when analyzing vascular resistance (Hoffman, 2011; Vanderpool & Naeije, 2018). To correct for blood viscosity, TPR and PASP were corrected for hematocrit (HCT). A reference HCT (HCTr) of 0.45 was used to normalize all measured TPR irrespective of their measured HCT (HCTm). The measured TPR was then divided by $e^{2(HCTr - HCTm)}$ (Vanderpool & Naeije, 2018). The HCT corrected TPR was multiplied by Q to obtain HCT

corrected PASP. The HCT corrected values of PASP and TPR are used throughout **Aim 3** (Chapter VI) of this dissertation.

Renal ultrasonography

Renal blood velocity was measured via Doppler ultrasound as previously described (Chapman *et al.*, 2020) during all interventions in **Aim 1** (Chapter IV) of this dissertation. The blood velocity of the renal vasculature was measured in the distal segment of the right renal artery. The same artery, for a given participant, was used throughout the experimental protocol. Renal blood velocity was measured across three consecutive cardiac cycles during which participants were instructed to perform a mid-exhalation, non-Valsalva breath hold lasting no more than 10 seconds. The same sonographer obtained all renal blood velocity measurements (Dr. Christopher Chapman, PhD). In addition, the location of the transducer was kept consistent by marking the participant with indelible ink.

Prior to the interventions in **Aim 1**, baseline measurements of renal blood velocity were obtained that were consistent with the body position during the interventions. For example, participants were in the supine position during COi, so all baseline measurements were made in the supine position. Participants were seated upright in the hot tub, so baseline measurements were made in an upright seated position. On the COi + HWI visit, two baseline periods, one seated and one supine, were performed to compare renal blood velocity measurements made during the interventions to the baseline in the same body position. During COi, renal blood velocity was measured at the end of the 4 minutes of hyperoxia, 5 minutes into the 10-minute CO rebreathe, and at the end of the 10 minutes of CO rebreathe for both tests. During HWI, renal blood velocity was measured every 5 minutes.

CALCULATION OF RENAL OXYGEN DELIVERY

For **Aim 1**, renal oxygen delivery was estimated to compare the strength of the stimuli during COi, HWI, and COi + HWI. Since renal oxygen delivery is a product of CaO_2 and Q_R (*equation 1, "Equations appendix"*), we used (100-HbCO)/100 as an approximation of CaO_2 and renal blood velocity as an approximation of Q_R since renal blood velocity and HbCO were the variables that changed with the interventions. Baseline HbCO was averaged from measurements made on the COi and COi + HWI visits and used to calculate the estimated resting CaO_2 for all three study visits. Measured baseline renal blood velocity was multiplied by the baseline estimated CaO_2 for all three visits to obtain the baseline estimate of renal oxygen delivery. For the COi + HWI visit, the seated baseline renal blood velocity was used in the calculation since the minimum renal blood velocity occurred during HWI in every participant, and we wanted to compare measurements in the same body position. For the COi and COi + HWI visits, peak HbCO was used to calculate the minimum estimated CaO_2. For the HWI visit, the baseline estimated CaO_2 was assumed to be unchanged. The minimum renal blood velocity measured during each intervention was multiplied by the minimum estimated CaO_2 to obtain the minimum estimation of renal oxygen delivery. An example calculation is provided in the *Calculations appendix*.

LUNG FUNCTION

Forced vital capacity

The forced vital capacity maneuver (FVC) measures the maximal amount of air that can be exhaled during a maximal expiratory effort following a maximal inspiration. It is a common clinical tool to assess adequate lung function and was performed according to societal standards

(Miller *et al.*, 2005). FVC was used as a screening tool for **Aim 3** (Chapter VI) of this dissertation.

During this test, participants breathed on a low-resistance mouthpiece with nose clips on. The mouthpiece was connected to a pneumotachograph, which had a space that allowed for a small sampling probe to be inserted that could measure volume and flow of the gas moving past it. The sampling probe was connected to a MedGraphics Elite Series Plethysmograph (Saint Paul, MN). To start, participants were seated comfortably upright with their feet flat on the ground and were asked to breathe normally for a minimum of 4 breaths. After at least 4 normal tidal breaths, participants were asked to maximally inhale as fast as possible to total lung capacity. They were immediately instructed to 'blast it out' and exhale maximally for at least six seconds until residual volume was reached. To achieve repeatability, a minimum of three similar tests were performed.

From this test we obtained maximal flow volume loops from which we could determine forced vital capacity (FVC). We also obtained the forced expiratory volume in the first second of expiration (FEV1), which can show flow limitations if one exists. The FEV1/FVC ratio is approximately 0.80 in most healthy adults. Lastly, we obtained the mid-expiratory flow (FEF25-75).

IV PLACEMENT & BLOOD DRAW PROCEDURE

IV catheter placement

Prior to blood draws and some ultrasound measures (see '*PFO screening*' above), an IV catheter was placed into a vein in the antecubital space. If a blood draw only required collection of vacutainer tubes, an alternative option was to use a straight needle attached to a vacutainer needle that could be inserted into a participant's vein.

Prior to placing an IV or straight needle, a tourniquet was instrumented around the participant's arm and the participant was instructed to rhythmically make a fist and relax. An appropriate peripheral vein in the antecubital space was identified visually and by palpating the area. The area was then thoroughly disinfected with an alcohol swab and the needle placed into the vein. If using an IV, adequate blood flow was tested by drawing back slightly on a syringe attached to the IV extension set, and if there was adequate blood flow, the IV was taped into place. Whenever the IV was not being actively used for a blood draw or for injection of saline bubbles, the extension set was flushed with sterile saline and closed off to prevent any foreign material from entering the IV.

Blood draw procedure

All blood for all studies was drawn into either serum separator vacutainer tubes (SSTs; gold or tiger top tubes pre-filled with a polyester-based gel with silica particles that act as a clot activator; Becton-Dickinson, Franklin Lakes, NJ), Ethylenediaminetetraacetic acid (EDTA; purple top tubes, Becton-Dickinson, Franklin Lakes, NJ)) coated vacutainer tubes, or heparinized syringes. Heparinized syringes were used to collect whole blood samples that were to be analyzed for HCT and co-oximetry, as heparin is an anti-clotting agent. To heparinize blood draw syringes, a tiny volume of heparin was drawn into the syringe via a fill needle. The volume of heparin was just sufficient to fill the reservoir tip of the blood draw syringe. SSTs were used to collect serum, and serum was used to measure inflammatory cytokine concentrations, EPO concentrations, iron concentration, ferritin concentration, and transferrin concentration. All SSTs contained a gel that was a density between that of serum and the additional components of blood (mostly red blood cells and some platelets and white blood cells), and this gel would end up between the serum and additional blood components after centrifugation. EDTA tubes were used

to collect whole blood that was used for white blood cell counts, as EDTA is also an anti-clotting reagent.

For all blood draws through an IV, a small amount of blood was drawn into a waste syringe to ensure no saline was being drawn into the blood collection tubes and/or syringes. After drawing waste, blood was drawn into the appropriate blood collection tubes and/or syringes. Following the blood draw, the IV catheter was fully flushed with sterile saline to ensure there were no clots that would form. If using a straight needle for blood draws into vacutainer tubes only, it was not necessary to draw waste, and the vacutainer tubes were each pressed against the vacutainer needle and held in place until filled.

BLOOD PROCESSING & ANALYSIS

Co-oximetry

Co-oximetry was performed on venous blood samples drawn into the pre-heparinized syringes. After drawing blood into the pre-heparinized syringe, it was immediately ready to be analyzed, or it was capped for later analysis. Co-oximetry was performed using the Radiometer OSM3 (Copenhagen, Denmark). To analyze a blood sample, the inlet flap of the OSM3 was opened and the syringe pressed against the sampling probe to form a tight seal. A tiny volume of blood was pushed through the sampling probe until sufficient sample was read, and then the inlet flap closed. The OSM3 provided total Hb (tHb) in g/dL, oxyhemoglobin (HbO_2), HbCO, methemoglobin (MetHb), and oxygen content (CO_2). The primary measure used from the OSM3 was HbCO. This was used to measure Hb mass (see '*Hb mass & COi*' above). Participants were also excluded from Hb mass and COi if their baseline HbCO was above 3% to prevent too great of an increase in HbCO post-COi, so co-oximetry was used as a screening tool as well.

HCT analysis

To analyze HCT, a small microcapillary tube was filled with blood from blood collected in a heparinized syringe. HCT was measured in at least duplicate. The microcapillary tubes were then spun in a centrifuge for 10 minutes at 10,000 rpm.

EPO analysis

EPO concentration was analyzed in the serum obtained from **Aim 1** (Chapter IV). To obtain serum for EPO analysis, venous blood drawn into SSTs was allowed to fully clot at room temperature for at least 30 minutes. After, it was spun in a centrifuge at 1500 g for 10 minutes. If the serum was not fully separated from the red blood cells after the initial spin, the blood was spun at the same speed for another five minutes. After the blood had been centrifuged, the serum was pipetted into pre-labeled crytotubes, and the cryotubes were stored in a -80°C freezer until analysis.

Frozen serum samples were thawed fully refrigerated on ice prior to analysis. EPO was analyzed via enzyme linked immunosorbent assay (ELISA) (BioLegend LEGENDMAX Human EPO ELISA,San Diego, CA, USA). Each participant had 22 different samples obtained across three study days (COi, HWI, and COi + HWI) that were analyzed in duplicate, so each participant was analyzed on their own plate with their own set of standard curves. However, all assays were the same lot number (Lot # B354852) and therefore had the same concentration of the top standard and identical protocols. The standard curve was generated by performing serial 1:2 dilutions of the top standard, which was 125 mIU/mL. A blank sample was also used. Per the manufacturer guidelines, serum samples were not diluted prior to analysis. To perform the assay, samples were first incubated on the pre-coated EPO antibody plate, and then incubated with a detection antibody. After forming the antibody 'sandwich', reagents were added to allow the

detection antibody to fluoresce. The plates were immediately analyzed via a plate reader (BioTek Synergy HT, Winooski, VT, USA). Samples were analyzed at both 450 and 570 nm, and the absorbance at 570 nm was subtracted from the absorbance at 450 nm. The difference in absorbance between the two wavelengths was used to calculate concentration based on a logarithmic standard curve. The sensitivity of the assay was 0.25 mIU/mL. All samples were above limits of detection of the assay except for one participant who was excluded from analyses (see '*Chapter IV'* below).

Inflammatory cytokine analysis

Serum from all dissertation aims (Chapters IV-VI) was analyzed for the concentration of 13 different inflammatory cytokines: interleukin (IL)-1β, interferon (IFN)-α2, IFN-γ, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. To obtain serum for cytokine analysis, venous blood drawn into SSTs was allowed to fully clot at room temperature for at least 30 minutes. After, it was spun in a centrifuge at 1500 g for 10 minutes. If the serum was not fully separated from the red blood cells after the initial spin, the blood was spun at the same speed for another five minutes. After the blood had been centrifuged, the serum was pipetted into pre-labeled crytotubes, and the cryotubes were stored in a -80°C freezer until analysis. Blood obtained from **Aim 3** was temporarily stored at -20°C while at the dive site, and after approximately 2 weeks was transferred to a -80°C freezer at the research facility until analyzed.

Frozen serum samples were fully thawed refrigerated on ice prior to analysis. Samples were analyzed using a multi-analyte flow assay kit (BioLegend LEGENDplex Human Inflammation panel 1, San Diego, CA, USA). The principal of the assay is nearly identical to that of an ELISA. However, rather than a plate that is pre-coated with the antibody specific to the one

protein in question, populations of capture beads with specific antibodies to the numerous proteins in question are used. The standard curve was generated by performing serial 1:4 dilutions of the top standard, concentrations of which were different for each analyte. A blank sample was also used. Serum samples were diluted twofold then incubated with 13 different capture beads. Following the first incubation, the samples were incubated with detection antibodies. The last incubation involved incubating samples with the chosen fluorophores, which were streptavidin (SA) and phycoerythrin (PE).

Following the assay procedure, all samples were analyzed via flow cytometry (BD Biosciences Accuri C6, Franklin Lakes, NJ, USA) according to manufacturer guidelines (Biolegend.com/legendplex). Samples were run on a low flow rate, which equated to a sampling rate of 14 μL/min and a core size of 10 mm. Beads were separated into two bead populations based on size. Within each different size bead population, the six or seven beads within that population were further separated based on fluorescence intensity. The slow flow rate allowed for adequate separation of bead populations without much background noise. The aim was to collect approximately 300 beads per analyte, or approximately 4,000 beads total. To ensure sufficient bead counts, approximately 5,500 beads were obtained during flow cytometry sampling.

All samples were run in duplicate and duplicates averaged. Upon analysis, any duplicate sample with a coefficient of variance (CV) > 30% was excluded from analysis, per suggestion in the manufacturer manual. Any sample with a high CV was re-run on a second assay, and those concentrations were used if the subsequent CV was < 30%. The lower limit of detection for each cytokine was defined as the mean plus 3 times the standard deviation of the mean fluorescence intensity of the blank. Samples below limits of detection were not excluded from analysis, and

concentrations were calculated from the extrapolated standard curve. The number of samples below limits of detection for each cytokine as well as the range of the lower limits of detection for each cytokine are provided in each individual chapter (Chapters IV – VI).

White blood cell count analysis

White blood cell counts were analyzed by QUEST Diagnostics (Secaucus, NJ, USA). Whole venous blood drawn into EDTA coated tubes was left at room temperature and did not require any additional processing. Whole blood was kept at room temperature for less than 48 hours before being analyzed by QUEST Diagnostics. Blood was analyzed via cell counting. White blood cell counts were measured for those that completed study 2 as a subset of **Aim 2** (Chapter V) of this dissertation.

Iron, ferritin, and transferrin analysis

Venous blood samples for iron, ferritin, and transferrin quantification were drawn into SSTs. Rather than centrifuging and pipetting serum into cryotubes and storing serum frozen on site, samples were instead kept at room temperature and analyzed by QUEST Diagnostics (Secaucus, NJ, USA) within 7 days. All samples were stable at room temperature for at least this amount of time. Iron was analyzed via spectrophotometry, ferritin via immunoassay, and transferrin via immunoturbidimetric assay.

CHAPTER IV

GREATER INCREASE IN ERYTHROPOIETIN IN FEMALES IN RESPONSE TO CARBON MONOXIDE INHALATION AND HOT WATER IMMERSION

This chapter was submitted to *The Journal of Physiology* with Dr. Christopher Chapman, Natasha E. Weiser, Emma R. Matsell, Kathryn M. Lucernoni, Samantha Chacon, Margaret M.B. Grivette, Dr. John R. Halliwill, Dr. Andrew T. Lovering, and Dr. Christopher T. Minson as co-authors. All experimental work was performed either by me independently or by C.L.C., N.E.W., E.R.M., K.M.L., S.C., and M.G.B.G. under my direction. The writing is entirely mine. All co-authors provided editorial assistance.

INTRODUCTION

When oxygen delivery to the kidneys is reduced in response to alveolar hypoxia, secretion of EPO from the kidneys markedly increases and returns to baseline once oxygen delivery is restored (Faura *et al.*, 1969; Abbrecht & Littell, 1972; Berglund *et al.*, 2002; Ge *et al.*, 2002; Jelkmann, 2011). The main function of EPO is to stimulate an increase in Hb mass, and more recently COi and heat stress have been utilized as interventions to target this pathway (Wang *et al.*, 2019*a*; Oberholzer *et al.*, 2019; Schmidt *et al.*, 2020; Rønnestad *et al.*, 2021, 2022; Lundby *et al.*, 2023). Acutely, COi (a single bolus of CO that raises HbCO 4-8%) has been shown to increase EPO, and chronic COi (3-4 weeks) can increase Hb mass (Wang *et al.*, 2019*a*; Schmidt *et al.*, 2020). Similarly, chronic heat stress (approximately 5 weeks) has been shown to increase Hb mass (Oberholzer *et al.*, 2019; Rønnestad *et al.*, 2021, 2022; Lundby *et al.*, 2023).

Despite studies showing that heat acclimation and chronic COi independently increase Hb mass, the studies examining the acute mechanisms by which these interventions work remain limited. To the best of our knowledge, no studies have found increases in EPO concentration in

response to an acute bout of heat stress, and very few studies have shown that acute COi can increase EPO concentration (Wang *et al.*, 2019*a*; Montero & Lundby, 2019; Schmidt *et al.*, 2020). Acute heat stress most likely stimulates EPO by causing renal vasoconstriction via increased renal sympathetic nerve activity, which reduces renal perfusion to support cutaneous vasodilation. The reduced renal perfusion would reduce renal oxygen delivery, thereby stimulating EPO secretion, but this has yet to be explored. Alternatively, COi reduces SaO_2 and therefore CaO_2, resulting in reduced renal oxygen delivery, but whether COi alters renal perfusion is unknown. Lastly, no studies have examined the effectiveness of combining acute COi and heat stress on EPO secretion, which plausibly leads to greater reductions in renal oxygen delivery via two different mechanisms and may provide an additive stimulus.

It is also important to note that there is a large degree of individual variability in EPO concentration in response to acute hypoxia, despite the same stimulus (Ge *et al.*, 2002; Baranauskas *et al.*, 2022). For example, changes in EPO concentration in response to moderate altitude (2800 meters) can range from -41 to 400% (Ge *et al.*, 2002). The reasons for this variability despite the same stimulus may be at least partially due to modulatory factors such as sex, concentrations of inflammatory cytokines, and/or iron status. The addition of inflammatory cytokines have been shown to reduce EPO concentration in response to hypoxia in cell culture models (Fandrey & Jelkmann, 1991; Faquin *et al.*, 1992; Jelkmann, 1998; La Ferla *et al.*, 2002), and low intracellular iron can suppress the transcription factor necessary for the production of EPO *in vitro* (Haase, 2013). Additionally, a significant knowledge gap exists regarding the characterization of these responses in females, because the few studies that have investigated EPO concentration in response to COi have been conducted only in males (Wang *et al.*, 2019*a*;

Montero & Lundby, 2019; Schmidt *et al.*, 2020), and only one study has shown that chronic heat acclimation can increase Hb mass in females (Lundby *et al.*, 2023).

Therefore, the purposes of this study were twofold. First, we sought to determine whether COi and HWI would increase EPO concentration and whether COi + HWI would augment EPO secretion. In addition, we sought to determine whether there were any sex differences in this response. A secondary aim was to determine whether inflammation or iron status were associated with the variability in EPO concentrations. Specifically, we hypothesized that 1) COi + HWI would augment increases in EPO concentration, 2) there would be no sex differences in EPO concentrations, and 3) those with the highest iron and lowest inflammation would have the greatest EPO concentrations in response to COi, HWI, and COi + HWI.

METHODS

Ethical approval and participant population

A total of 21 individuals volunteered to participate in this study after being advised both verbally and in writing to the nature of the experiments. Participants signed an informed consent form to participate in the study approved by the University of Oregon Research Compliance Services (protocol STUDY00000189). All studies were performed in accordance with the standards set forth by the *Declaration of Helsinki* except for registration in a database. Of the 21 individuals who volunteered for the study, 4 participants were lost to follow-up communication. Of the 17 participants that completed data collection, one participant had EPO concentrations below limits of detection for all time points during all interventions and was therefore excluded from analyses (see '*EPO analysis'* below). Therefore, data are presented for 16 participants. There were equal numbers of females and males (n = 8 females). All participants identified as recreationally active and were free from self-reported cardiovascular and renal diseases. To the

best of their knowledge, no participants reported as being descendants from major high-altitude populations (Aymaran, Tibetan, or Ethiopian); however, one female participant was born in Colorado (elevation ~5,500' or 1675 meters). The remaining participants were all born in low altitude cities. In addition, none of the participants traveled to altitudes greater than 2000 meters for greater than 6 hours of time in the month leading up to the study.

Study design and instrumentation

Participants completed one screening visit followed by three interventional study visits in a balanced and randomized crossover design, illustrated in Figure 4. The interventions were COi, HWI, and COi + HWI. Given the acute nature of the study, each intervention was separated by at least one week to avoid any compounding effects of the interventions (e.g., heat acclimation). All participants started the study visits in the morning between 06:30 and 08:30.

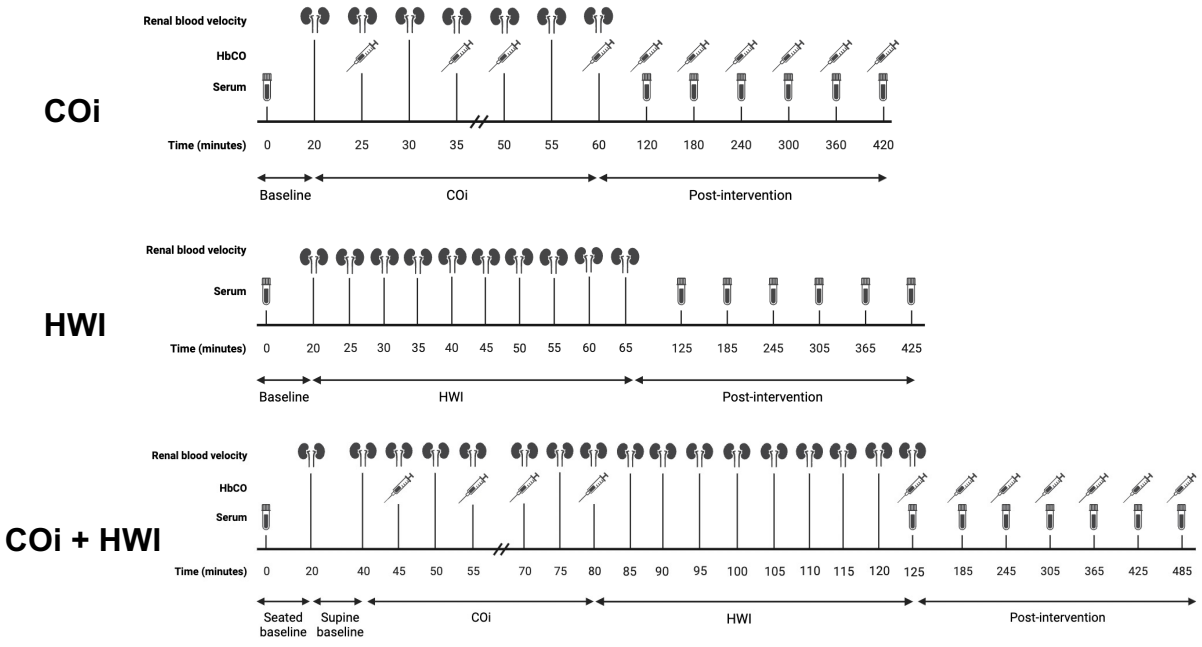


Figure 4. Overview of the study design for Aim 1. Participants completed three interventions in a randomized, balanced, crossover design. Data collected included renal

blood velocity measures and venous blood samples for measurement of HbCO, cytokine concentrations, and EPO concentration.

Upon arrival on all study visits, participants provided a urine sample for measurements of urine specific gravity. All participants had urine specific gravity <1.020 or were asked to consume 5mL/kg body weight of water if ≥ 1.020. Afterwards, participants were instrumented with an IV catheter for blood draws. In addition, participants were instrumented with a blood pressure cuff to measure brachial artery blood pressure during the interventions (SunTech Medical Tango M2 Stress Test Monitor, Morrisville, NC). On the days that involved HWI, participants were instrumented with either a rectal temperature probe (ZOLL Medical Corporation YSI Reusable rectal temperature probe, Chelmsford, MA; n = 8) or a core temperature pill (HQInc CorTemp Sensor, Palmetto, FL; n = 8) for monitoring core temperature. If a participant opted to use a core temperature pill, they were asked to ingest the pill approximately 10 hours before they began their study visit. Participants had nude weight measured before and after HWI. After instrumentation, baseline measurements were made (see "Measurements" below), and then participants completed the assigned intervention. Subsequently, participants had blood drawn from the IV catheter once every hour for 6 hours post-intervention. On the COi + HWI visit, participants had one additional post-intervention blood draw immediately after HWI (0 hr time point), which corresponded to 1 hour after COi. Participants were permitted to leave the lab but were instructed not to exercise between blood draws.

Interventions

For the COi visit, participants completed duplicate Hb mass measurements via the 10-minute optimized CO rebreathe technique (Siebenmann *et al.*, 2017). Participants began by

resting supine for 20 minutes for normalization of plasma volume shifts due to gravity (Hagan *et al.*, 1978; Keiser *et al.*, 2013). After 20 minutes of supine rest, we obtained baseline measurements (see "Measurements" below). Afterwards, participants breathed 100% oxygen for 4 minutes followed by rebreathing a small bolus of CO in 100% oxygen for 10 minutes. The volume of CO was determined based on participant weight (0.8mL/kg body weight for females and 1.0 mL/kg body weight for males) (Siebenmann *et al.*, 2017). After the first Hb mass test, participants completed a duplicate Hb mass test, which was intended to raise the peak HbCO to 10-15%.

For the HWI visit, participants sat upright in a hot tub heated to 40°C for 45 minutes. Prior to entering the hot tub, participants rested in a seated upright position for 20 minutes in the laboratory (temperature = 23.0 ± 0.8°C; humidity = 37.3 ± 10.3%). After 20 minutes of upright rest, we obtained baseline measurements (see "Measurements" below). While in the hot tub, we sought to increase core temperature to 38.5°C with water immersion up to the sternum. In the event that core temperature of 38.5°C was reached (n = 4), water immersion up to the waist was performed and a fan turned on in an attempt to clamp core temperature at 38.5°C to improve participants' thermal perception at this elevated core temperature. All participants were given 2mL of water per kg of body weight after 25 minutes in the hot tub.

On the COi + HWI visit, participants completed the above interventions back-to-back. COi occurred first, followed by HWI to ensure a quick transition between interventions. Baseline data was obtained prior to either intervention in both an upright position as well as supine. This allowed us to compare measurements during the intervention to the baseline data in the same body position.

Measurements

Renal blood velocity, mean arterial pressure, and renal vascular resistance. Renal artery blood velocity was measured via Doppler ultrasound before and during every intervention as previously described (Chapman *et al.*, 2020, 2023). Briefly, renal blood velocity was measured in the distal segment of the right renal artery (renal artery) using the coronal approach. The same artery, for a given participant, was used throughout the experimental protocol. Measurements were taken in the supine position (Chapman *et al.*, 2023) for the COi visit and were taken in the seated position during the HWI visit to replicate the position of participants in the hot tub. During the COi + HWI visit, measurements were first taken in the seated position following 20 minutes of rest and were then taken in the supine position following another 20 minute rest. This was deemed necessary so that changes in renal blood velocity could be effectively compared to the same body posture at baseline within a given intervention. During visits involving HWI, a clear surgical sleeve (CIVCO Medical Solutions, Kalona, IA) was used to cover the transducer and allow for underwater renal blood velocity measurements. Renal blood velocity was measured across three consecutive cardiac cycles during which participants were instructed to perform a mid-exhalation, non-Valsalva breath hold lasting no more than 10 seconds. The same sonographer obtained all renal blood velocity measurements (C.L.C.). In addition, the location of the transducer was kept consistent by marking the participant with indelible ink.

During COi, renal blood velocity was measured during the final minute of the 4 minutes of 100% oxygen, 5 minutes into the 10-minute CO rebreathe, and during the final minute of the 10-minute CO rebreathe for both tests. While in the hot tub, renal blood velocity was measured every 5 minutes. Due to the difficulty of this measurement, a small number of renal blood velocity data points are missing (n=2 on the COi visit; n=0 on the HWI visit; n=1 on the COi +

HWI visit). Brachial artery blood pressure was measured at the same time points. Renal vascular resistance was calculated as mean arterial pressure/renal blood velocity.

Venous blood samples. An IV catheter was placed into an antecubital vein for obtaining blood draws on all visits, including screening. On the screening visit, participants had 15 mL of blood drawn for analyses of iron, ferritin, and transferrin. Blood samples were drawn into serum separator tubes (SSTs; gold or tiger top tubes pre-filled with a polyester-based gel with silica particles that act as a clot activator; Becton-Dickinson, Franklin Lakes, NJ),) and kept at room temperature to be analyzed within 7 days (QUEST Diagnostics, Secaucus, NJ). Iron was analyzed via spectrophotometry, ferritin via immunoassay, and transferrin via immunoturbidimetric assay.

On the intervention study visits, prior to the interventions and every hour after the intervention for 6 hours, a 15 mL venous blood sample was drawn from the IV catheter into SSTs (Becton-Dickenson, Franklin Lakes, NJ). Baseline blood samples from all three interventions were used to measure cytokine concentrations, and blood samples from all time points and all interventions were used to measure EPO concentration. Due to IV issues, a small number of blood samples were unobtainable: 1) HWI visit, n=3 total samples (n=1 at hour 1, n=1 at hour 2, and n=1 at hour 6) and 2) COi+HWI visit, n=3 total samples (n=1 at hour 1, n=1 at hour 3, and n=1 at hour 6). All venous blood samples drawn on the intervention visits for cytokine and EPO analyses were allowed to sit at room temperature for at least 30 minutes to fully clot, after which they were centrifuged at 1500g for 10 min. Serum was separated and frozen at -80°C until analyzed.

Blood analysis

Cytokine analysis. Frozen serum samples were fully thawed, refrigerated on ice. Only baseline samples from each of the three interventions were analyzed for cytokine concentrations. Three different plates were used for cytokine analysis, but all three samples from a given participant were analyzed on the same plate with the same standard curve. Samples were analyzed using a multi-analyte flow assay kit as described in the manual (BioLegend LEGENDplex Human Inflammation panel 1, San Diego, CA). Briefly, samples were incubated with 13 different capture beads: IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Samples were diluted twofold prior to analysis. All samples were analyzed via flow cytometry (BD Biosciences Accuri C6) according to manufacturer guidelines (Biolegend.com/legendplex). All samples were run in duplicate, and duplicates were averaged. Any duplicate sample with a coefficient of variance (CV) > 30% was excluded from analysis, per manufacturer recommendations. Table 1 documents the number of samples excluded for this reason. The lower limit of detection for each cytokine was defined as the mean plus 3 times the standard deviation of the mean fluorescence intensity of the blank. Samples below limits of detection were included in the analysis. The calculated concentration based on the extrapolated standard curve was used for the samples below limits of detection. The number of samples below limits of detection for each cytokine as well as the range of the lower limits of detection from the 3 different plates for each cytokine are listed in Table 1.

EPO analysis. EPO was analyzed via ELISA (BioLegend LEGENDMAX Human EPO ELISA, San Diego, CA), with assay sensitivity of 2.5 mIU/mL. Frozen serum samples were fully thawed, refrigerated on ice. Per the manufacturer guidelines, sampleSan Ies were not diluted prior to analysis. All samples were above limits of detection of the assay except for one

participant (noted above in "Ethical approval and participant population") who was excluded from analyses.

Calculation of estimated renal oxygen delivery index

To compare the strength of the stimulus between the COi and HWI visits, we estimated renal oxygen delivery. Since renal oxygen delivery is a product of CaO_2 and Q_R, we used (100-HbCO)/100 as an approximation of CaO_2 and renal blood velocity as an approximation of Q_R since these variables changed with the interventions. Baseline HbCO was averaged from measurements made on the COi and COi + HWI visits and used to calculate the estimated resting CaO_2 for all three study visits. Measured baseline renal blood velocity was multiplied by the baseline estimated CaO_2 for all three visits to obtain the baseline estimate of renal oxygen delivery. For the COi + HWI visit, the seated baseline renal blood velocity was used in the calculation since the minimum renal blood velocity occurred during HWI in every participant, and we wanted to compare measurements in the same body position. For the COi and COi + HWI visits, peak HbCO was used to calculate the minimum estimated CaO_2. For the HWI visit, the baseline estimated CaO_2 was assumed to be unchanged. The minimum renal blood velocity measured during each intervention was multiplied by the minimum estimated CaO_2 to obtain the minimum estimation of renal oxygen delivery.

Table 1. Inflammatory cytokine data from Aim 1.

		COi				HWI				COi + HWI			
	LLOD range	n included	Mean ± SD	n with CV>30%	n < LLOD	n included	Mean ± SD	n with CV>30%	n < LLOD	n included	Mean ± SD	n with CV>30%	n < LLOD
IL-1β (pg/mL)	0.86 - 1.97	16	38.52 ± 44.39	0	1	16	38.05 ± 51.98	0	3	16	34.95 ± 41.06	0	0
IFN-α2 (pg/mL)	0.92 - 1.63	16	36.78 ± 30.83	0	0	16	35.15 ± 30.34	0	0	16	35.49 ± 28.12	0	0
IFN-γ (pg/mL)	1.88 - 3.59	16	40.52 ± 50.02	0	2	16	39.28 ± 53.27	0	4	16	34.33 ± 45.00	0	1
TNF-α (pg/mL)	0.64 - 3.87	16	78.10 ± 100.30	0	2	16	75.51 ± 94.37	0	1	16	70.86 ± 82.62	0	2
MCP-1 (pg/mL)	5.70 - 7.08	16	663.70 ± 266.60	0	0	15	664.50 ± 235.30	1	0	16	701.00 ± 215.30	0	0
IL-6 (pg/mL)	0.49 - 2.47	16	32.95 ± 29.91	0	0	16	27.22 ± 25.77	0	1	16	25.27 ± 19.85	0	0
IL-8 (pg/mL)	13.91 - 23.20	16	166.70 ± 146.90	0	0	16	137.80 ± 139.60	0	1	16	163.40 ± 128.50	0	0
IL-10 (pg/mL)	1.30 - 4.06	16	34.38 ± 40.64	0	2	16	27.58 ± 39.60	0	3	16	28.63 ± 42.24	0	2
IL-12p70 (pg/mL)	1.28 - 2.91	16	24.11 ± 30.69	0	1	16	23.07 ± 32.12	0	2	16	21.45 ± 29.56	0	0
IL-17A (pg/mL)	0.24 - 0.78	16	6.24 ± 4.59	0	0	16	6.31 ± 5.54	0	1	16	5.84 ± 4.79	0	0
IL-18 (pg/mL)	2.40 - 11.22	16	676.60 ± 138.3	0	0	16	612.80 ± 142.70	0	0	16	635.70 ± 184.90	0	0
IL-23 (pg/mL)	3.66 - 10.41	16	52.95 ± 80.38	0	3	16	55.96 ± 85.08	0	3	16	55.65 ± 83.79	0	1
IL-33 (pg/mL)	10.96 - 98.13	16	625.10 ± 578.10	0	1	16	577.90 ± 543.00	0	1	16	569.60 ± 513.00	0	0

Samples were analyzed using three assays. The lowest and highest lower limit of detection (LLOD) from the three assays is provided (LLOD range). On each study day, the mean ± standard deviation (SD), number of observations included in the analysis (n included), number of observations with a CV greater than 30% (n with CV > 30%), and number of observations below LLOD (n < LLOD) are provided.

Data and statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9.4.1) with p *a priori* set to equal 0.05. A one-way mixed-effects analysis with Sidak post hoc was performed to compare baseline EPO concentrations from each participants' first, second, and third interventional visits. HbCO, renal blood velocity, renal vascular resistance, mean arterial pressure, estimated renal oxygen delivery, and EPO concentrations were analyzed over time and between interventions via a 2-way mixed effects analysis with Sidak post-hoc.

The change in EPO concentrations were analyzed over time and between sex within each interventional visit via a 2-way mixed effects analysis with Sidak post-hoc. To determine if there were sex differences in the stimulus variables within each of the three study visits (peak and Δ HbCO, minimum and Δ renal blood velocity, and maximum and Δ renal vascular resistance), unpaired t-tests were used. Additionally, the mimimum and change in estimated renal oxygen delivery were analyzed between interventions and sex via a 2-way mixed effects analysis with Sidak post-hoc.

Simple linear regressions were used to test whether there were significant relationships between iron variables (iron, ferritin, and transferrin) and baseline cytokine concentrations with peak and changes in EPO concentrations on each study visit. Because cytokines are rarely released alone and exhibit a large degree of redundancy and pleiotropy (Ozaki & Leonard, 2002), we created a multiple linear regression model to predict EPO concentrations from all 13 cytokines. To avoid the issue of multicollinearity in our model, we performed principal component analysis (PCA) followed by principal component regression (PCR) using parallel analysis as the method to choose which components to use in the model. Baseline cytokine concentrations on each visit were reduced to one component (COi: eigenvalue of 8.864

accounted for 68.18% of the variability; HWI: eigenvalue of 9.704 accounted for 74.65% of the variability; COi + HWI: eigenvalue of 9.058 accounted for 69.67% of the variability). These components were then regressed onto the peak EPO concentration and change in EPO concentration for each of the three interventions.

RESULTS

EPO concentrations in response to COi, HWI, and COi + HWI

There was an effect of the study visit on baseline EPO concentration ($p = 0.0419$), but there were no pairwise differences when comparing baseline EPO concentrations on the first (5.78 ± 3.42 mIU/mL), second (6.15 ± 3.93 mIU/mL), and third (7.59 ± 4.98 mIU/mL) visits. EPO concentration increased over time in all three visits, and the pattern of this response did not differ between interventions (Fig 5A). When comparing only baseline and peak EPO concentrations, all three interventions increased EPO from baseline to peak concentration, and there were no differences between the interventions (Fig 5B).

Sex differences in EPO concentrations

Interestingly, the increase in EPO was driven entirely by females. There was an interaction between sex and time on the change in EPO concentration for the COi (Fig 6A), HWI (Fig 6B), and COi + HWI (Fig 6C) visits. Females increased EPO concentration on all three study visits while males did not (Fig 6). In addition, females had a significantly greater change in EPO concentration than males 5- and 6-hours post-COi (Fig 6A).

Iron, inflammation, & EPO

Of the iron and immune variables measured, transferrin was the only variable that predicted EPO concentration across all three study visits (Table 2). However, the relationship was a positive linear relationship, indicating that lower iron availability in the form of high

transferrin resulted in a greater EPO increase in response to the interventions. Neither ferritin, iron, nor any of the baseline cytokine concentrations predicted the peak concentration or change in EPO (Table 2). Principal component regression results indicate that baseline inflammation did

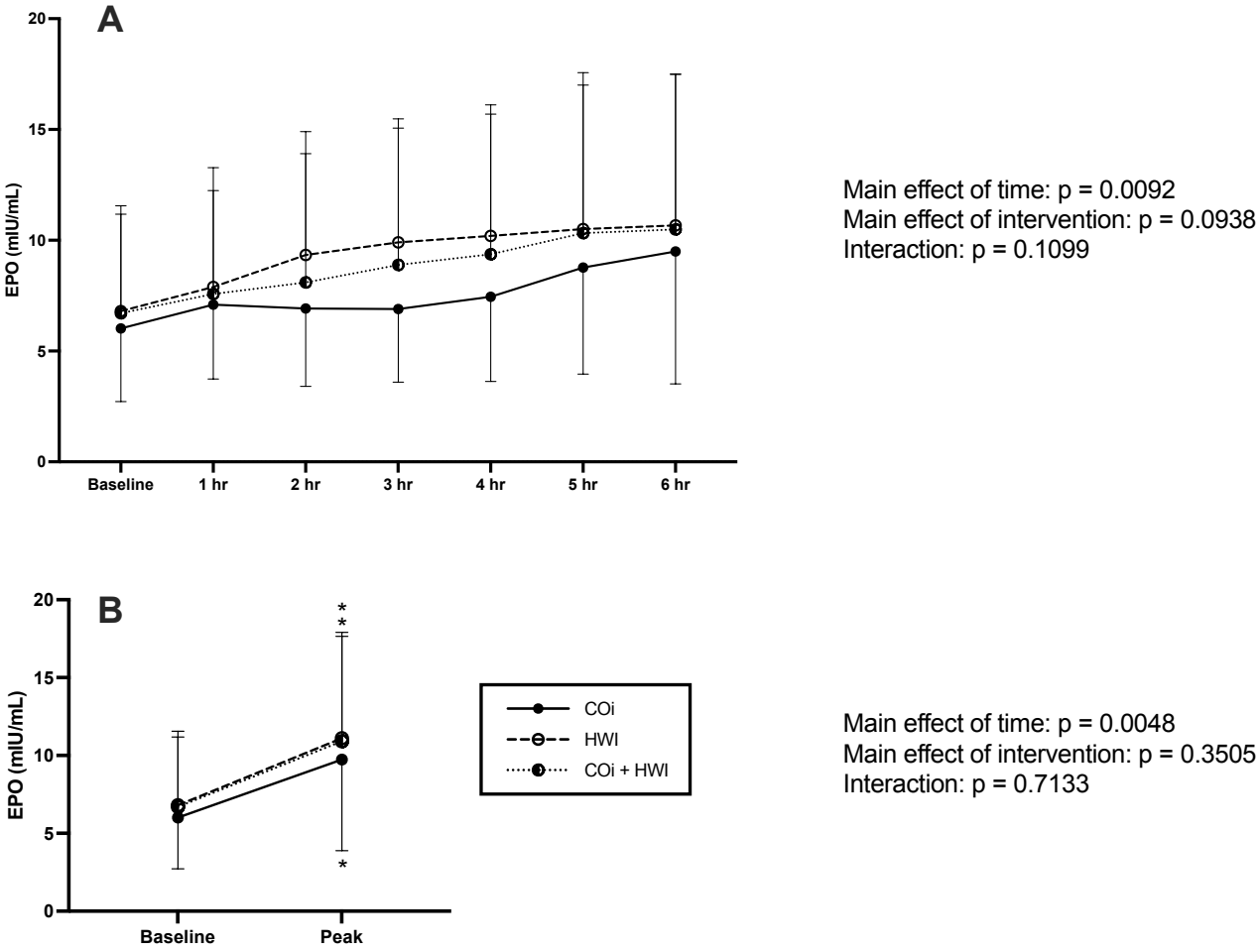


Figure 5. Comparing EPO concentration across the interventions. A) EPO concentration over time on COi, HWI, and COi + HWI visits. B) EPO concentration from baseline to peak concentration on COi, HWI, and COi + HWI visits. COi = closed circles, solid lines; HWI = open circles, dashed lines; COi + HWI = half-filled circles, dotted lines. p-values for main effects and interactions are provided. * denotes significant pairwise comparisons ($p < 0.05$) compared to baseline within each intervention.

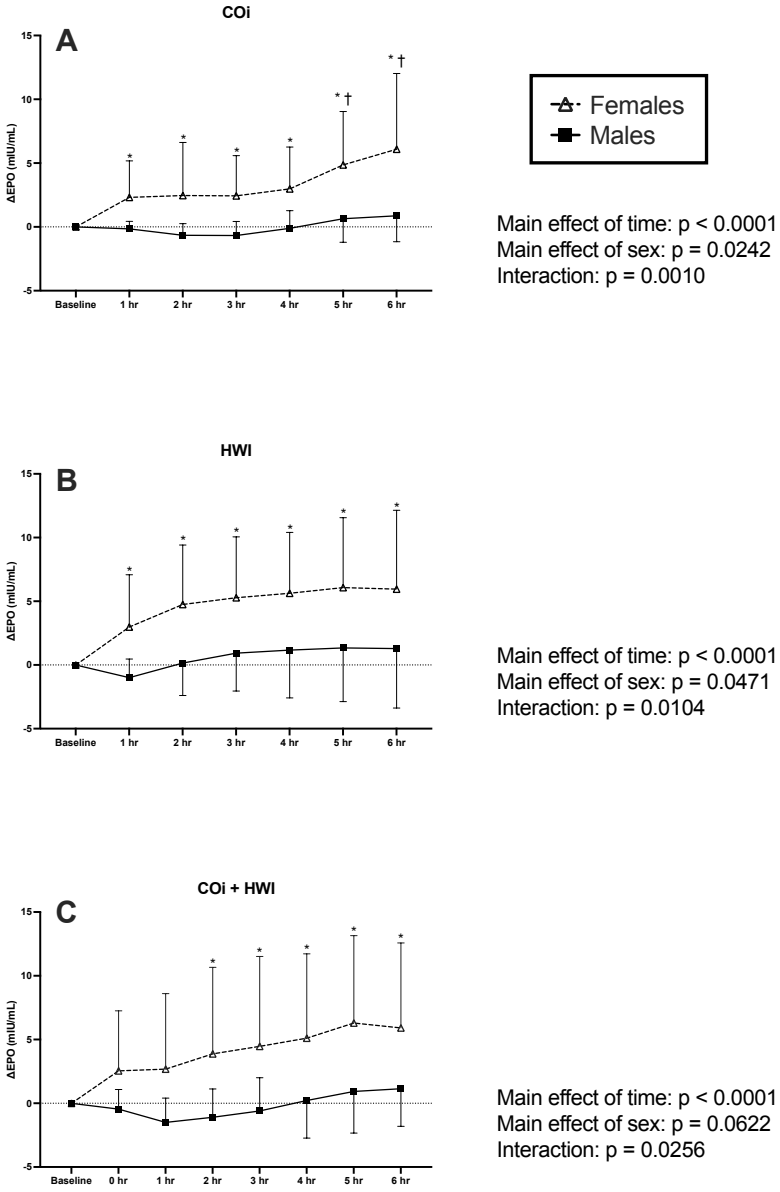


Figure 6. Sex differences in the change in EPO. Females (open triangles, dashed lines) increased EPO from baseline whereas males (closed squares, solid lines) did not in response to all three interventions: A) COi, B) HWI, and C) COi + HWI. The change in EPO was calculated as the EPO concentration at that time point minus the baseline concentration. p-values for main effects and interactions are provided. * denotes significant pairwise comparisons ($p < 0.05$) compared to baseline within a sex; † denotes significant pairwise comparisons ($p < 0.05$) comparing females to males at that time point.

Table 2. Relationships between immune & iron variables with EPO.

	COi						HWI						COi + HWI					
	Peak EPO			ΔEPO			Peak EPO			ΔEPO			Peak EPO			ΔEPO		
	Slope	R^2	p-value	Slope	R^2	p-value	Slope	R^2	p-value	Slope	R^2	p-value	Slope	R^2	p-value	Slope	R^2	p-value
Iron (mcg/dL)	-0.0515	0.1326	0.1656	-0.0607	0.2604	0.0434 *	0.0008	0.0000	0.9986	-0.0248	0.0368	0.4768	-0.0400	0.0560	0.3775	-0.0541	0.1685	0.1142
Ferritin (ng/mL)	-0.0609	0.1845	0.0969	-0.0516	0.1878	0.0935	-0.0486	0.0940	0.2481	-0.0543	0.1760	0.1057	-0.0563	0.1106	0.2082	-0.0586	0.1972	0.0849
Transferrin (mg/dL)	0.1050	0.7235	< 0.0001 *	0.0880	0.7199	< 0.0001 *	0.0713	0.2663	0.0407 *	0.0712	0.3990	0.0087 *	0.1022	0.4800	0.0029 *	0.0962	0.7005	< 0.0001 *
IL-1β (pg/mL)	-0.0440	0.1117	0.2058	-0.0200	0.0327	0.5028	-0.0265	0.0441	0.4349	0.0033	0.0010	0.9061	-0.0660	0.1501	0.1381	-0.0419	0.1000	0.2328
IFN-α2 (pg/mL)	-0.0512	0.0730	0.3116	-0.0333	0.0436	0.4379	-0.0605	0.0787	0.2927	-0.0234	0.0177	0.6229	-0.0603	0.0589	0.3650	-0.0551	0.0810	0.2854
IFN-γ (pg/mL)	-0.0277	0.0564	0.3760	-0.0130	0.0175	0.6257	-0.0246	0.0402	0.4567	-0.0034	0.0012	0.8994	-0.0274	0.0311	0.5133	-0.0177	0.0213	0.5895
TNF-α (pg/mL)	-0.0174	0.0984	0.2607	-0.0109	0.0493	0.4087	-0.0216	0.0972	0.2399	-0.0065	0.0132	0.6722	-0.0298	0.1239	0.1812	-0.0209	0.1003	0.2320
MCP-1 (pg/mL)	0.0032	0.0208	0.5946	-0.0003	0.0002	0.9600	-0.0067	0.0566	0.3931	-0.0034	0.0221	0.6968	0.0046	0.0199	0.6020	-0.0007	0.0009	0.9144
IL-6 (pg/mL)	-0.0430	0.0484	0.4129	0.0214	0.0169	0.2406	-0.0384	0.0228	0.5765	0.0471	0.0516	0.3975	-0.0754	0.0459	0.4259	0.0083	0.0009	0.9117
IL-8 (pg/mL)	-0.0137	0.1186	0.1915	-0.0089	0.0708	0.3193	-0.0147	0.0989	0.2355	-0.0059	0.02360	0.5700	-0.0158	0.0848	0.2738	-0.0099	0.0552	0.3813
IL-10 (pg/mL)	-0.0409	0.0808	0.2861	-0.0076	0.0039	0.8180	-0.0344	0.0432	0.4397	0.0090	0.0044	0.8068	-0.0418	0.0639	0.3447	-0.0190	0.0216	0.5867
IL-12p70 (pg/mL)	-0.0357	0.0352	0.4867	-0.0006	0.0000	0.9891	-0.0365	0.0322	0.5064	0.0203	0.0149	0.6524	-0.0610	0.0665	0.3349	-0.0218	0.0141	0.6618
IL-17A (pg/mL)	-0.3275	0.0660	0.3368	-0.2194	0.0419	0.4468	-0.3825	0.1046	0.2217	-0.2039	0.0448	0.4316	-0.3583	0.0603	0.3593	-0.1899	0.0279	0.5362
IL-18 (pg/mL)	-0.0067	0.0248	0.5602	0.0062	0.0307	0.5165	-0.0060	0.0168	0.6319	0.0036	0.0094	0.7209	0.0179	0.2249	0.0634 †	0.0139	0.2221	0.0654
IL-23 (pg/mL)	-0.0214	0.0868	0.2680	-0.0152	0.0622	0.3518	-0.0249	0.1046	0.2216	-0.0103	0.0267	0.5453	-0.0296	0.1258	0.1776	-0.0178	0.07481	0.3053
IL-33 (pg/mL)	-0.0030	0.08682	0.2679	-0.0017	0.0392	0.4623	-0.0043	0.1265	0.1764	-0.0012	0.0159	0.6422	-0.0046	0.1135	0.2020	-0.0035	0.1109	0.2075

The slope, R^2, and p-value of the regression between each predictor and the peak and change (Δ) in EPO concentration are provided for each of the three interventions. p-values presented represent whether the slope of the regression is significantly non-zero. * indicates $p < 0.05$.

Stimulus for EPO secretion

The stimulus data does not fully explain why COi + HWI did not augment EPO, nor does the stimulus data fully explain the sex differences in EPO. As expected, HbCO increased following COi and was identical on the COi and COi + HWI visits (Fig 7A). HWI reduced renal blood velocity and mean arterial pressure and increased renal vascular resistance on both the HWI and COi + HWI visits (Fig 7B-D), while COi did not change these variables (Fig 7E-G). All three interventions reduced estimated renal oxygen delivery from baseline (Fig 8). Baseline estimated renal oxygen delivery was not different between interventions, but the minimum estimated renal oxygen delivery was lower on the HWI and COi + HWI visits compared to COi (Fig 8). However, the minimum estimated renal oxygen delivery was not different between the COi + HWI and HWI visits (Fig 8).

Additionally, there were no sex differences in the minimum or change in estimated renal oxygen delivery for each of the three interventions (Fig 9A & 9B). Similarly, there were no sex differences in the minimum or change in renal blood velocity or the maximum or change in renal vascular resistance for each of the three interventions (Table 3). However, females did have a greater peak and change in HbCO on both the COi and COi + HWI visits (Table 3).

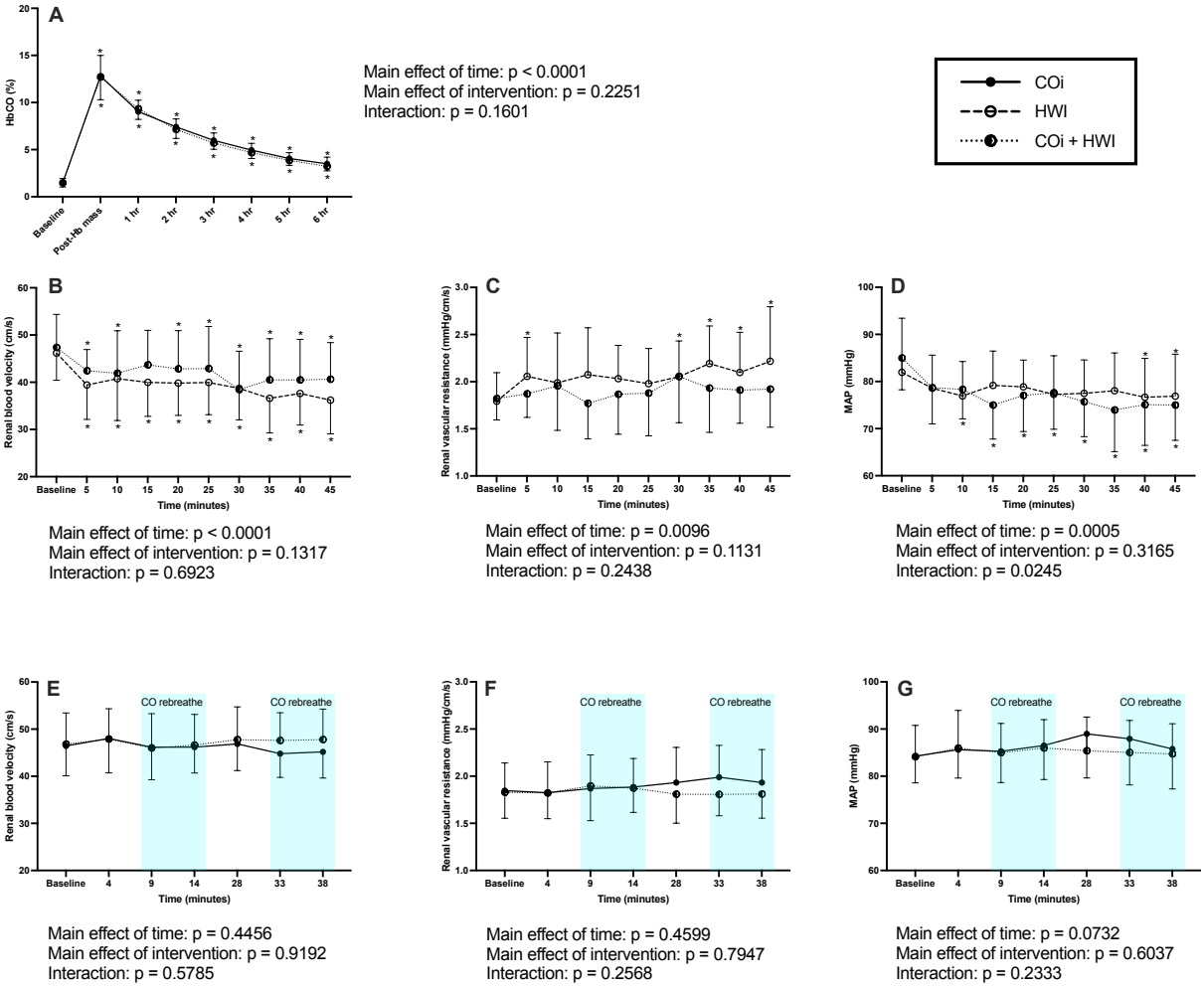


Figure 7. Stimuli for EPO production. A) HbCO on the COi and COi + HWI visits. B) renal blood velocity on the HWI and COi + HWI visits. C) renal vascular resistance on the HWI and COi + HWI visits. D) mean arterial pressure on the HWI and COi + HWI visits. E) renal blood velocity on the COi and COi + HWI visits. F) renal vascular resistance on the COi and COi + HWI visits. G) mean arterial pressure on the COi and COi + HWI visits. COi = closed circles, solid lines; HWI = open circles, dashed lines; COi + HWI = half-filled circles, dotted lines. p-values for main effects and interactions are provided. For panels E-G, shaded bars indicate the measurements taken during CO rebreathe. * denotes significant pairwise comparisons ($p < 0.05$) compared to baseline within each intervention

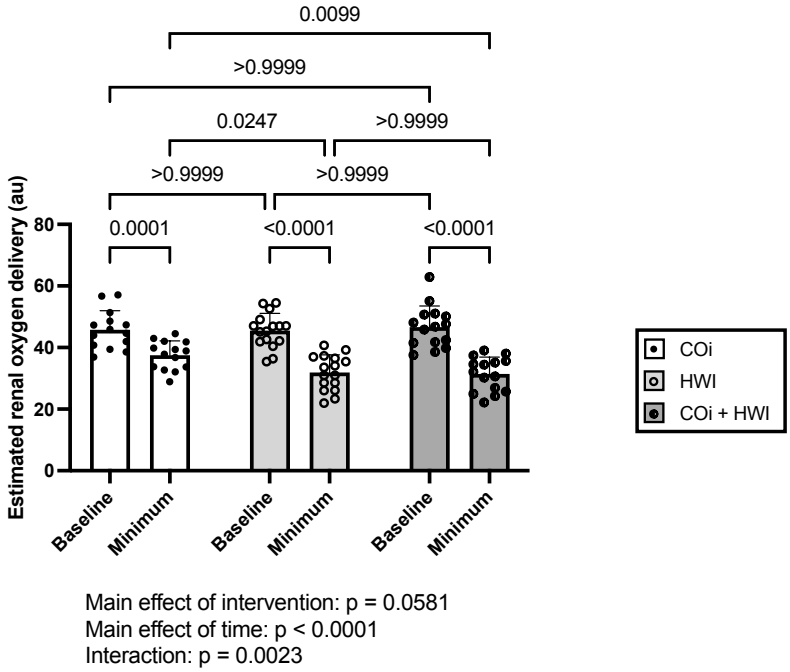


Figure 8. Estimated renal oxygen delivery. Baseline estimated renal oxygen delivery and minimum renal oxygen delivery achieved during each intervention (COi = white bars, closed circles; HWI = light grey bars, open circles; COi + HWI = dark grey bars, half-filled circles). Brackets indicate pairwise comparisons, and the values above the brackets indicate p-values of the comparisons. p-values for main effects and interactions are provided.

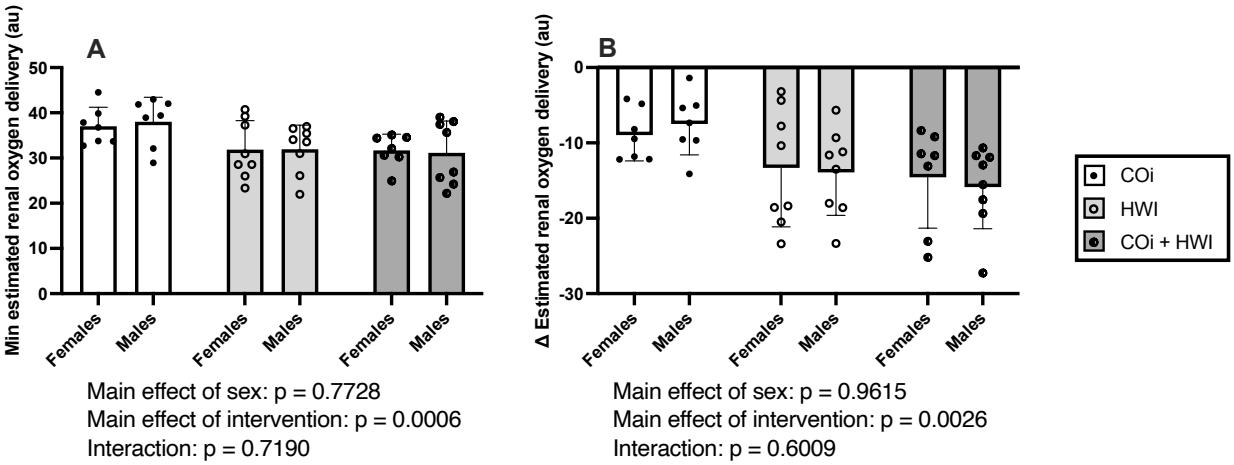


Figure 9. Sex differences in estimated renal oxygen delivery. A) Minimum estimated renal oxygen delivery in females and males during each intervention. B) The change in estimated renal oxygen delivery in females and males during each intervention. COi = white bars, closed circles; HWI = light grey bars, open circles; COi + HWI = dark grey bars, half-filled circles. p-values for main effects and interactions are provided.

Table 3. EPO stimulus data in females and males.

		Females	Males	p-value
COi	Peak HbCO (%)	13.96 ± 2.35	11.66 ± 1.34	0.0300 *
	Δ HbCO	12.48 ± 2.43	10.19 ± 1.07	0.0287 *
	Min renal blood velocity (cm/s)	43.23 ± 4.98	43.10 ± 6.60	0.9679
	Δ Renal blood velocity	-3.42 ± 3.61	-3.15 ± 4.16	0.8957
	Max renal vascular resistance (cm/s/mmHg)	1.84 ± 0.33	1.68 ± 0.24	0.3329
	Δ Renal vascular resistance	-0.04 ± 0.17	-0.13 ± 0.21	0.4155
HWI	Min renal blood velocity (cm/s)	32.31 ± 6.64	32.45 ± 5.48	0.9646
	Δ Renal blood velocity	-13.49 ± 7.92	-14.12 ± 5.84	0.8582
	Max renal vascular resistance (cm/s/mmHg)	1.73 ± 0.36	1.74 ± 0.34	0.90503
	Δ Renal vascular resistance	0.02 ± 0.50	-0.13 ± 0.35	0.4981
COi + HWI	Peak HbCO (%)	14.0 ± 2.75	11.42 ± 1.06	0.0270 *
	Δ HbCO	12.63 ± 2.80	9.85 ± 0.89	0.0181 *
	Min renal blood velocity (cm/s)	36.84 ± 4.13	35.18 ± 8.02	0.6293
	Δ Renal blood velocity	-10.12 ± 7.07	-12.57 ± 5.86	0.4772
	Max renal vascular resistance (cm/s/mmHg)	1.46 ± 0.22	1.64 ± 0.22	0.1450
	Δ Renal vascular resistance	-0.34 ± 0.22	-0.20 ± 0.24	0.2614

The mean ± standard deviation of the females and males are provided. Changes in stimulus values were calculated as the baseline value subtracted from the maximum or minimum value. p-values represent comparisons between females and males. * indicates $p < 0.05$.

DISCUSSION

In the present study we found that COi and HWI, both independently and when combined, increased EPO concentration. However, contrary to our hypothesis, the combination of COi and HWI did not further augment the increased EPO concentrations above either COi or HWI alone. An additional important finding was that the increase in EPO observed was driven by the female participants, and males did not increase EPO. The reasons for the observed sex differences are unknown but unlikely to be completely explained by sex differences in the stimulus. Additionally, it is intriguing that the males in the present study did not increase EPO

concentration in response to COi, which contradicts several previous studies (Wang *et al.*, 2019*a*; Montero & Lundby, 2019; Schmidt *et al.*, 2020). Reasons for these contradictory observations will be examined in greater detail in the subsequent subsections. Lastly, we found that iron and inflammation did not predict peak EPO concentrations or the change in EPO concentrations in response to COi and HWI independently and in combination.

Effects of COi and HWI on EPO concentration

All three interventions significantly increased EPO concentration from baseline, which is expected as both interventions reduced estimated renal oxygen delivery, and this is presumably the mechanism causing increased EPO. We confirmed results from other studies that show that COi alone can increase EPO concentration (Wang *et al.*, 2019*a*; Montero & Lundby, 2019; Schmidt *et al.*, 2020). However, to the best of our knowledge we are the first to show that heat alone can acutely increase EPO concentration in humans since other studies have shown no increase in EPO in response to acute heat stress (Akerman *et al.*, 2017; Oberholzer *et al.*, 2019).

Despite finding that all three interventions stimulated an increase in EPO concentration, combining COi and HWI did not augment EPO secretion. This was contrary to our hypothesis given that COi and HWI presumably target different components of renal oxygen delivery, specifically Q_R and CaO_2. However, COi + HWI may not have provided a truly additive stimulus for EPO secretion. Estimated renal oxygen delivery was lower during COi + HWI than it was on the COi visit, indicating that COi + HWI was a more potent presumptive stimulus than COi alone. However, COi + HWI did not significantly reduce estimated renal oxygen delivery below that of HWI, which supports the conclusion that COi + HWI may not have provided an additive stimulus. Still, the estimated stimulus data do not fully explain the observation that EPO increased to the same extent from all interventions, as we would expect to see lower EPO

concentration in response to COi if the response was entirely dependent on the estimated stimulus. This likely indicates that either we are not fully capturing the stimulus for EPO secretion and/or EPO can be produced in response to a stimulus that is in addition to changes in renal oxygen delivery.

One limitation to the present study is that renal doppler can only reliably measure renal blood velocity and not renal artery diameter, which is needed to calculate Q_R. It is generally assumed that the diameter of the renal artery does not change during acute interventions such as HWI (Conboy *et al.*, 2010; Chapman *et al.*, 2020), but we were not able to confirm this. Similarly, we did not obtain arterial blood samples, so we were unable to directly measure CaO_2 and instead had to estimate CaO_2 with HbCO. Additionally, we did not measure variables such as renal metabolic rate, oxygen extraction, or oxygen utilization, which may have altered the true stimulus for EPO secretion. Therefore, it is possible that the indirect measures obtained in the present study did not fully capture the stimulus for EPO secretion.

Additionally, while the kidney is the primary site of EPO production in the adult human, there are numerous extrarenal sites of EPO production. The liver is the primary site of EPO production during embryonic development and can still produce EPO in the adult human (Haase, 2010). In addition, EPO mRNA has been shown to be expressed in most other tissue types, including the brain, lungs, heart, bone marrow, spleen, and reproductive tract (Haase, 2010). It is possible that we did not account for extrarenal stimuli and that extrarenal EPO production contributed to our findings.

Because COi + HWI did not reduce estimated renal oxygen delivery below that of HWI, it is possible that hypoxia and heat have overlapping mechanisms of action, which could lead to them being antagonistic or hypo-additive in their effects on EPO concentration rather than

additive (Lloyd & Havenith, 2016). We chose COi and HWI because we wanted to provide stimuli that did not overlap in their mechanisms of action, but it is nevertheless possible that there is more overlap in how heat and hypoxia affect the kidney than previously understood. For example, higher core temperatures during HWI will right shift the oxyhemoglobin dissociation curve, reducing SaO_2 and therefore CaO_2. It was previously unknown whether COi would alter Q_R, but we confirmed that there was no effect of COi on renal blood velocity or renal vascular resistance. Therefore, if there are additional overlapping mechanisms of action between COi and HWI independent of heat's effect on CaO_2, it is currently unknown.

Although we are the first to examine this non-augmented EPO concentration in response to COi + HWI, other studies have shown that heat and other forms of hypoxia do not provide additive stimuli. One study found that EPO concentration was not significantly different in post-exercise trials in which participants cycled in either heat and normobaric hypoxia (FiO_2 of 14.5%) or heat only (Hayashi *et al.*, 2020), further supporting the results from the present study. Chronically, combining live-high-train-low with heat acclimation did not increase exercise performance beyond that achieved with heat acclimation training only (McCleave *et al.*, 2017). Lastly, one study examined acute exercise performance decrements in response to hypoxia and heat and found that although combining heat and hypoxia worsened exercise performance below that of heat alone, combining heat and hypoxia did not reduce exercise performance below that of hypoxia alone (Bradbury *et al.*, 2019). These studies, combined with the present data, indicate that heat and hypoxia likely do not provide an additive stimulus to augment EPO secretion, and future work should explore the potential overlapping mechanisms of action between these interventions.

Sex differences in EPO

We found that females had a significant increase in EPO in response to each intervention and that males did not increase EPO in response to any of the three interventions. One potential explanation for why females had a greater increase in EPO in response to COi is that females had a higher peak HbCO on both visits involving COi. Therefore, on the COi and COi + HWI visits, the higher HbCO in females likely provided a greater stimulus for EPO secretion. However, this does not explain why there were differences in the change in EPO concentration between females and males on the HWI visit. There were no differences between females and males in any stimulus variable on the HWI visit that could explain the sex differences in EPO in response to HWI, so the reasons for the observed sex differences in EPO in response to HWI are unknown.

Additionally, it is important to speculate why we did not see an increase in EPO concentration in males in response to COi, which has been shown by others (Wang *et al.*, 2019*a*; Montero & Lundby, 2019; Schmidt *et al.*, 2020). In the study by Schmidt *et al.* (2020), EPO concentration did not appear to be drastically different between the COi and placebo groups until 16 hours after COi. However, EPO was only measured in a subset of participants (n = 3) every 8 hours, so it is difficult to directly compare to the present study. Wang *et al.* (2019) and Montero & Lundby (2019) both found that COi increased EPO concentration within 6 hours, which is more comparable with the timing in our study. The discrepancies between these two studies and the present study are most likely explained by the stimulus. Montero & Lundby (2019) raised HbCO to approximately 18% while the peak HbCO in the males in the present study averaged approximately 11.5%, indicating that the CO stimulus in the present study was less severe. While the CO stimulus provided by Wang *et al.* (2019) (HbCO of approximately 4.4%) was lower than

in the present study, they also utilized exercise training immediately post-COi, which could have augmented the stimulus. Additionally, EPO only increased approximately 1 mIU/mL (Wang *et al.*, 2019*a*), which is comparable to our study in which EPO increased approximately 1 mIU/mL in males in response to COi after 6 hours.

Interestingly, in a meta-analysis aimed to study variability in Hb mass increases in response to altitude training camps, males were found to respond better to altitude training camps, as measured by a greater increase in Hb mass (Nummela *et al.*, 2021). Additionally, females and males similarly increased Hb mass in response to heat acclimation (Lundby *et al.*, 2023). Those studies did not present EPO data, so it is unknown whether there were any differences in EPO concentrations between females and males. However, it is interesting to speculate that although males may have a blunted EPO secretion in response to acute stimuli compared to their female counterparts, functionally they may require a lower EPO concentration to significantly increase Hb mass. Despite females having greater increases in EPO concentrations in response to our acute interventions, chronic use of these interventions may not result in a greater increase in Hb mass in females compared to males. In support of this, in hemodialysis patients, females required a greater exogenous EPO dose to maintain Hb concentration in a healthy range compared to males (Coronado Daza & Cuchi, 2019). This supports the idea that a greater change in EPO or a greater concentration of EPO may be necessary to increase Hb mass in females. Therefore, there are possible differences between females and males in the translation from EPO to viable red blood cells, although this remains largely unstudied.

In addition to sex, it is worth considering other factors that could contribute to heterogeneity of the participants in the present study. There may be differences in EPO due to

factors such as race and/or birthplace that could affect EPO. There are known racial differences in EPO responsiveness, with Black hemodialysis patients requiring higher doses of exogenous EPO to maintain Hb mass (Lacson *et al.*, 2008). Additionally, there may be a hyporesponsiveness in EPO production due to high altitude birthplace. High altitude population studies show blunted responses to hypoxia in Tibetan and Ethiopian natives (Petousi *et al.*, 2014; Bigham, 2016) given that these populations have long-term generational adaptations as a result of high birth altitude. As noted in the methods, one participant was born in a high-altitude city in Colorado, and the participant excluded for having EPO concentrations below limits of detection at all time points was Black. Therefore, heterogeneity within our participant pool may explain some additional variability in EPO concentration.

The relationships between EPO, iron, and inflammation

The present study found that regardless of whether individual cytokines were considered or all cytokines were considered together, there were no significant linear relationships between inflammatory cytokine concentrations and either the peak or change in EPO concentration on all three study days. Data from multiple cell culture studies suggest that higher concentrations of inflammatory cytokines, primarily IL-1β, IFN-γ, and TNF-α would blunt EPO production in response to hypoxia while high IL-6 would promote it (Fandrey & Jelkmann, 1991; Faquin *et al.*, 1992; Vannucchi *et al.*, 1994; La Ferla *et al.*, 2002). We speculate that our differing results may be related to our use of a human model, only studying baseline cytokine concentrations, or the relative concentrations of cytokines. It is likely that in otherwise healthy humans, baseline cytokine concentrations are not expressed at high enough concentrations to disrupt EPO and that higher concentrations like those measured under pathological conditions are required to disrupt EPO production. In support of this idea that higher cytokine concentrations are needed to inhibit

EPO production, studies have shown that EPO inhibition is dose-dependent such that cells treated with the highest cytokine concentrations, particularly, IL-1β, TNF-α, and IFN-γ, had the greatest EPO inhibition (Faquin *et al.*, 1992; Vannucchi *et al.*, 1994).

We found a positive linear relationship between baseline transferrin and the change in EPO concentration on all three study days. During hypoxia, hypoxia inducible factor (HIF)-2 stabilization will increase EPO but will also increase bioavailability of iron in order to make more red blood cells (Haase, 2013). In addition, erythropoiesis will inhibit hepcidin to increase iron availability for erythropoiesis (Ganz, 2007). However, intracellular iron can provide negative feedback on this process, as low intracellular iron concentrations were found to suppress HIF-2α translation (Haase, 2013), thereby reducing EPO production. Therefore, in humans who have low iron, EPO hyporesponsiveness to hypoxia may be partially explained by iron status. However, the positive linear relationship between transferrin and EPO that we observed refutes our hypothesis given that high transferrin is indicative of low iron availability, which would theoretically suppress EPO. Importantly, the individuals driving this associative relationship are a subset of female participants who had the highest transferrin that responded to the interventions with the greatest change in EPO.

Conclusions & ethical implications

Overall, this study shows that COi and HWI can increase EPO, although there is variability in who responds to these interventions. A significant observation was that there were no differences in EPO concentration between COi and HWI. Even though COi can increase EPO, it is nevertheless potentially dangerous, particularly if not used in well controlled environments. Our results emphasize that HWI is an equally effective alternative therapy that can

be used by those wishing to increase circulating EPO concentration but without the risks associated with COi.

Our data also suggest that these interventions, at least acutely, are more effective in females. This may be partially due to an augmented stimulus in females in response to COi, but the reasons for the sex differences in EPO in response to HWI are still unknown. Therefore, future work should examine mechanisms underlying the sex differences in EPO. Lastly, baseline iron and inflammation likely do not play a modulatory role in EPO production, so future work should examine other potential factors that can explain inter-individual variability in EPO.

CHAPTER V

ASSOCIATIONS OF HEMOGLOBIN MASS WITH INFLAMMATION, IRON STATUS, AND PATENT FORAMEN OVALE IN FEMALES AND MALES

This chapter was submitted to *American Journal of Physiology – Heart and Circulatory Physiology* with Aaron W. Betts, Karleigh E. Bradbury, Emma R. Matsell, Tyler Kelly, Elizabeth A. Gideon, Dr. John W. Duke, and Dr. Andrew T. Lovering as co-authors. All experimental work was performed either by me independently or by A.W.B., K.E.B., E.R.M., and T.K. under my direction. The writing is entirely mine. All co-authors provided editorial assistance.

INTRODUCTION

Adequate tissue oxygen delivery is dependent upon having a sufficient number and concentration of red blood cells. Accordingly, making this measurement is of physiologic importance and is done by quantifying the total Hb mass (Siebenmann *et al.*, 2017). However, there is a large degree of variability in what are considered normal Hb mass values, likely suggesting there are a multitude of factors influencing or modulating Hb mass. Some of this variability can be explained by sex assigned at birth, as it is well documented that females have significantly lower Hb concentration and Hb mass when expressed in absolute (grams) or relative (grams/kg of body weight) units than males (Murphy, 2014; Handelsman *et al.*, 2018; Falz *et al.*, 2019). Likewise, it has been shown that lean body mass has the strongest correlation with Hb mass (Falz *et al.*, 2019; Goodrich *et al.*, 2020), and this is responsible for most of the inter-individual variability in Hb mass. However, while normalizing Hb mass to lean body mass can eliminate some variability (Falz *et al.*, 2019; Goodrich *et al.*, 2020), at a given lean body mass there is still variability in Hb mass of approximately 300 grams within the same sex

(Goodrich *et al.*, 2020), which can represent roughly 25-60% of an individual's total Hb mass. Therefore, it is important to understand what other factors may modulate Hb mass and explain the observed substantial individual variability.

One potential modulator of Hb mass is the immune system, which can have negative effects on erythropoiesis and therefore Hb mass (Cooper *et al.*, 2003; Nandakumar *et al.*, 2016). In fact, in clinical conditions characterized by elevated concentrations of circulating cytokines and white blood cells, anemia of inflammation can occur (Fraenkel, 2017; Weiss *et al.*, 2019; Ganz, 2019). The immune system can affect Hb mass via numerous pathways, including impairing proliferation and/or differentiation of HSCs and erythroid precursor cells (Mamus *et al.*, 1985; Raefsky *et al.*, 1985; Schooley *et al.*, 1987; Wang *et al.*, 1995; Tarumi *et al.*, 1995; Xiao *et al.*, 2002; Felli *et al.*, 2005; Libregts *et al.*, 2011; McCranor *et al.*, 2014; Pietras *et al.*, 2016; Etzrodt *et al.*, 2019; Swann *et al.*, 2020), impairing Hb synthesis (McCranor *et al.*, 2014), reducing the lifespan of red blood cells (Libregts *et al.*, 2011), and promoting the recycling of mature red blood cells (Lu *et al.*, 2020; Xu & Huang, 2020). However, the associations between circulating cytokines and white blood cells with Hb mass in healthy humans is unknown.

Additionally, iron availability, which is essential to the formation of Hb, may also be a key modulator of Hb mass. Iron deficiency is the leading cause of anemia, particularly in females (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; Cappellini *et al.*, 2020). Furthermore, in the context of iron availability, it is important to consider iron storage and transport in the form of ferritin and transferrin, respectively, in addition to total blood iron. Low ferritin and high transferrin can be indicative of reduced iron availability even in the face of normal total blood iron, and together these factors may play a modulatory role in baseline Hb mass by limiting Hb synthesis.

An additional factor that may influence Hb mass is the presence of a PFO. A PFO is a remnant structure of the fetal circulatory system present in approximately 25-40% of the adult population (Hagen *et al.*, 1984; Marriott *et al.*, 2013; Elliott *et al.*, 2013) that acts as an intracardiac right-to-left shunt (Duke *et al.*, 2020). Accordingly, it is reasonable to speculate a PFO presents a mechanism of increased arterial hypoxemia, which could result in a compensatory increase in Hb mass. However, despite studies showing those with a PFO have worse gas exchange efficiency compared to those without a PFO at sea level, there is no evidence the presence of a PFO alone results in arterial hypoxemia in otherwise healthy humans (Lovering *et al.*, 2011; Duke *et al.*, 2020), and thus the PFO is unlikely to cause a sufficient magnitude of hypoxemia to drive an increased erythropoietic response. However, some considerations that have yet to be studied are differences in immune system function and iron regulation in those with a PFO. There are data to suggest iron deficiency and the presence of a PFO are both linked to stroke (Shovlin, 2014; Topiwala *et al.*, 2021), but whether there is a direct link between a PFO and iron deficiency, and how this relationship alters Hb mass, is unknown. It is also unknown if those with a PFO have altered expression of inflammatory cytokines, so this needs to be explored as a potential source of variability in Hb mass.

Taken together, the primary purpose of this study was to determine whether various immune system components and/or iron status parameters are related to Hb mass. Likewise, we were interested in whether or not the presence of a PFO affected sea level Hb mass and whether the presence of a PFO altered the relationships between immune system components and/or iron status parameters with Hb mass. an Hb mass. Additionally, those with a PFO would have lower Hb mass, and this would be largely driven by those with a PFO having lower iron availability.

METHODS

Ethical approval

Participants that completed the present study are pooled from three larger studies with a variety of aims that all involved Hb mass testing and venous blood draws. All protocols were approved by the University of Oregon Research Compliance Services (Protocol numbers STUDY00000189, STUDY 00000174, and STUDY 00000019). Participants signed an informed consent form specific to the study or studies they participated in. All studies were performed in accordance with the standards set forth by the *Declaration of Helsinki* except for registration in a database. Some participants participated in multiple studies, and for those participants, replicate data were averaged. Figure 10A provides an overview of how many participants participated in each study and how many participants participated in multiple studies.

Study design

Study 1. The primary aim of study 1 was to examine EPO concentrations in response to COi and HWI. As part of the screening process for this study, participants had venous blood samples drawn to quantify iron, ferritin, and transferrin, but white blood cell counts and the presence or absence of a PFO was not assessed. Following screening, there were three experimental visits each separated by one week. Two of the experimental visits from this study involved Hb mass testing, and Hb mass data were averaged from those two visits. In addition, baseline blood samples were taken on all three experimental visits and used for measurements of inflammatory cytokines (see "*Cytokine analysis"* below for the list of measured cytokines). Inflammatory cytokine concentrations were averaged from all three visits. All testing was completed within approximately 2 hours in the morning (start time between 06:30 and 08:30). Blood samples were taken immediately before any interventional testing.

Study 2. The primary aim of study 2 was to determine thermoregulatory mechanisms that may be responsible for PFO related differences in core temperature previously reported (Davis *et al.*, 2015, 2017). A sub-aim of study 2 was to determine differences in Hb mass between those with and without a PFO as part of the current work here. As part of the screening process for this study, participants had venous blood samples drawn for measurements of iron, ferritin, and transferrin. Participants did not have white blood cell counts measured. All participants had PFO screening performed as part of this study. On a separate experimental day, venous blood was drawn prior to and after 1 hour of submaximal exercise for measurements of inflammatory cytokines. Baseline inflammatory cytokine concentrations alone were used for the present study. Baseline inflammatory cytokines were measured in the morning (approximately 07:15). Some female participants participated in two study days where venous blood was drawn pre- and post-exercise to account for menstrual cycle phases (or high/low hormone phases of birth control), so for those participants, baseline cytokine data were averaged from both experimental days. Because there are potential immune fluctuations throughout the menstrual cycle (Oertelt-Prigione, 2012), we opted to take an average of cytokine concentrations in both menstrual cycle phases rather than assume one phase or the other was representative of a participant's baseline. Hb mass was measured on a different day than blood draws, and Hb mass was not restricted by time of day.

Study 3. The overarching aim of study 3 was to determine the effect of exogenous iron and EPO treatment on Hb mass and exercise performance at a moderate altitude. As part of the screening process for this study, participants had venous blood samples drawn for quantification of iron, ferritin, transferrin, and white blood cell counts. All participants had PFO screening performed as part of this study. On a separate day, Hb mass was measured, and a second venous

blood sample was taken for measurements of inflammatory cytokine concentrations. All testing occurred prior to any drug interventions. Hb mass measurements were not restricted by the time of day. Blood for cytokine analysis was drawn immediately prior to Hb mass testing and was similarly unrestricted by time of day.

Measurements

Measurements made in the present study included PFO screening, Hb mass quantification, and venous blood draws for quantification of iron and immune variables. Because participants were drawn from multiple studies, not every participant completed every measurement. In addition, some participants withdrew from their respective study prior to completing all testing, but already had Hb mass measured at the time of their withdrawal and are therefore included in the study. Figure 10B provides details on how many participants completed each measurement, which are described in detail below.

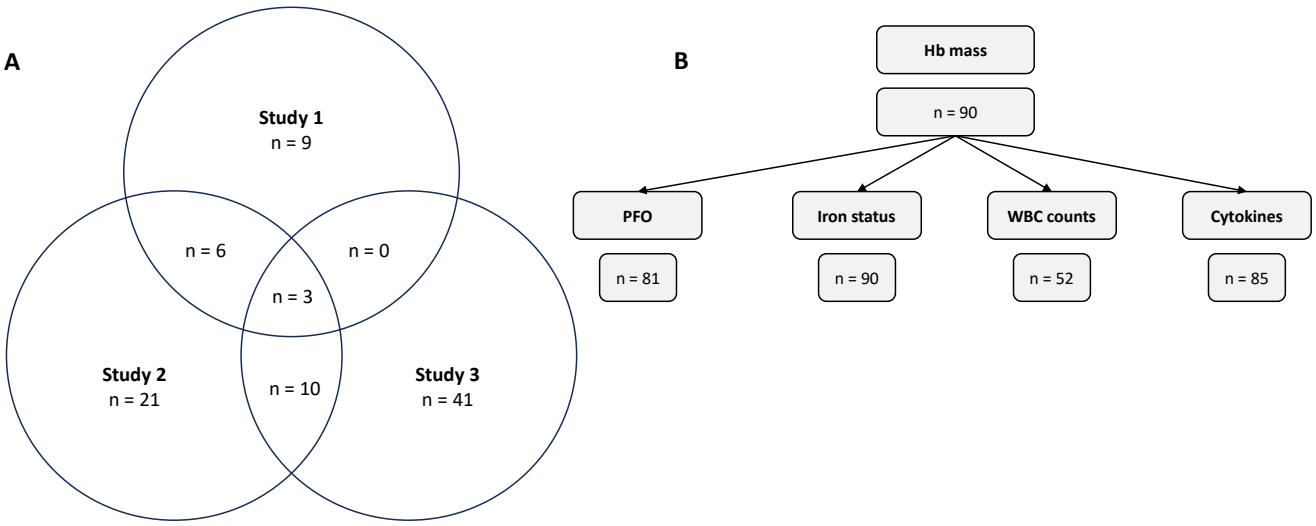


Figure 10. Study overview for Aim 2. A) The number of participants who participated in each of the three studies individually, as well as the number of participants who

participated in multiple studies. B) The number of data points for each of the four modulatory variables examined in the present study. WBC = white blood cell.

PFO screening. An initial echocardiography (Philips ie33) screening and bubble study was performed on participants in studies 2 and 3, but not study 1, as previously published (Lovering & Goodman, 2012; Elliott *et al.*, 2013). Briefly, an IV catheter was placed in a peripheral vein for injection of saline contrast with and without a Valsalva maneuver to confirm the presence or absence of a PFO. The release of a Valsalva maneuver is not required to open a PFO but instead creates conditions where right atrial pressure transiently exceeds left atrial pressure as bubbles appear in the right side of the heart while we are simultaneously imaging so that we can see if bubbles travel to the left side of the heart during these optimal conditions. We considered a PFO to be present if bubbles appeared in the left ventricle ≤3 cardiac cycles following right heart opacification with saline contrast. Participants with no bubbles detected in the left ventricle were considered to have no PFO and were assigned to the PFO- group. Participants with bubbles in the left ventricle within 3 cardiac cycles were considered to have a PFO and assigned to the PFO+ group. Those with bubbles appearing in the left ventricle in greater than 3 cardiac cycles were considered to have no PFO and assigned to the PFO- group.

Hemoglobin (Hb) mass test. All Hb mass tests were done in duplicate on the same day via the 10-minute CO rebreathe technique (Siebenmann *et al.*, 2017). Briefly, an IV catheter was placed into a peripheral vein in the participant's arm. Then, participants laid supine for 20 minutes with feet elevated approximately 20 cm for normalization of plasma volume due to gravitational shifts (Keiser *et al.*, 2013). Next, participants breathed 100% oxygen from an open circuit for 4 minutes. At the end of 4 minutes, a 2mL venous blood sample was drawn into a

heparinized syringe and analyzed for HbCO (Radiometer OSM3). After, participants were switched to a closed circuit via a manual sliding valve (Series 2870, Hans Rudolph, Shawnee, KS, USA), and a small bolus of CO was injected based on body weight (0.8 mL/kg for females; 1.0 mL/kg for men) (Siebenmann *et al.*, 2017). This volume was chosen to increase HbCO by approximately 4-7% for one test, which is sufficient to measure changes in HbCO while being a safe concentration of CO for participants. The rebreathe circuit was pre-filled and occasionally filled as needed with 100% oxygen during the rebreathe period, and the circuit also contained a CO_2 scrubber to prevent hypercapnia. After 10 minutes of CO rebreathing, another venous blood sample was drawn into a heparinized syringe and analyzed for HbCO (Radiometer OSM3). The volume of gas remaining in the rebreathe circuit and the concentration of CO (ppm) was measured (CO-220 Carbon Monoxide Meter, Fluke, Everett, WA). Participants then repeated the 4 minutes of 100% oxygen and 10 minutes of CO breathing. Duplicate tests were averaged. All duplicate tests had an error less than 3% to be included in the study. The average percent error between duplicate tests was 0.89%. Absolute Hb mass was measured in grams (g), and relative Hb mass was measured in grams relative to body weight (g/kg).

Venous blood draws & processing. Venous blood was drawn via an IV catheter and/or a venipuncture for measurement of iron, ferritin, transferrin, white blood cell counts, and inflammatory cytokines. Blood for iron, ferritin, and transferrin was drawn into serum separator tubes (SSTs; gold or tiger top tubes pre-filled with a polyester-based gel with silica particles that act as a clot activator; Becton-Dickinson, Franklin Lakes, NJ) and kept at room temperature, and were subsequently analyzed via QUEST Diagnostics (Secaucus, NJ) within 6 days of being drawn. Whole venous blood for white blood cell counts was drawn into an EDTA-coated tube (purple top tube, Becton-Dickinson, Franklin Lakes, NJ) and kept at room temperature, and

blood was analyzed via QUEST Diagnostics (Secaucus, NJ) within 2 days of being drawn. Lastly, blood for inflammatory cytokine analysis was drawn into SSTs (gold or tiger top tubes pre-filled with a polyester-based gel with silica particles that act as a clot activator; Becton-Dickinson, Franklin Lakes, NJ). Blood was allowed to sit for at least 30 minutes to fully clot. After, the blood was spun at 1500g for 10 minutes and serum frozen at -80°C until analysis.

Blood analysis

Venous blood was analyzed for iron, ferritin, and transferrin concentrations by QUEST Diagnostics (Secaucus, NJ, USA). Iron was analyzed via spectrophotometry, ferritin via immunoassay, and transferrin via immunoturbidimetric assay. White blood cell counts were also measured by QUEST Diagnostics (Secaucus, NJ, USA) via cell counting.

To measure inflammatory cytokine concentrations, serum samples were first thawed while fully refrigerated on ice. Samples were analyzed using a multi-analyte flow assay kit as described in the manual (BioLegend LEGENDplex Human Inflammation panel 1, San Diego, CA, USA). Briefly, samples were incubated with 13 different capture beads: IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Samples were diluted twofold prior to analysis. All samples were analyzed via flow cytometry (BD Biosciences Accuri C6, Franklin Lakes, NJ, USA) according to manufacturer guidelines (Biolegend.com/legendplex). All samples were run in duplicate and averaged. All samples were analyzed with 7 different assays all from the same lot number (Lot #B347171) with the same top standard concentrations.

Upon analysis, any duplicate sample with a coefficient of variance (CV) >30% was excluded from analysis, as suggested by the manufacturer. There were two participants' samples that each had one analyte with a CV >30% (n = 1 for MCP-1; n = 1 for IL-18), but both of those

participants participated in multiple studies, so cytokine concentrations were taken from those samples instead and the analytes with high CVs were excluded. Therefore, in the 85 participants included in inflammatory cytokine analysis, there were no missing data points due to high CVs (Table 4). Additionally, any cytokine concentrations below limits of detection were included in the analysis. Limits of detection were defined as three times the standard deviation of the mean fluorescence intensity of the blank. Table 4 documents the number of samples which were below limits of detection for each cytokine for each of the six study days on which cytokine concentrations were analyzed. These values below limits of detection were calculated using the extrapolated standard curves.

Statistical analysis

All variables (height, weight, age, absolute and relative Hb mass, iron, ferritin, transferrin, concentrations of all 13 inflammatory cytokines, and white blood cell counts) were analyzed with an ordinary 2-way ANOVA with PFO status and sex as independent variables. Alpha set *a priori* to be or less than 0.05. Even though the main purpose of this study was not to examine sex differences in Hb mass and its potential modulatory variables, there are known sex differences in Hb mass, iron regulation, and the immune system (Klein & Flanagan, 2016; Falz *et al.*, 2019; Cappellini *et al.*, 2020), so we included sex as a factor to avoid drawing conclusions about the effect of a PFO that were masked by sex differences. Post-hoc testing was performed using the Sidak post-hoc analysis to examine differences between PFO status within each sex.

To determine whether there were linear relationships between iron and immune variables with absolute and relative Hb mass, simple linear regressions were performed. P-values presented represent whether the slope of the relationship was significantly non-zero. In addition, simple linear regressions were performed to determine whether the presence or absence of a PFO

altered the slope or intercept of the relationships between iron and immune variables with absolute and relative Hb mass.

In addition to simple linear regressions, we created 3 multiple linear regression models that were used predict Hb mass. Iron, ferritin, and transferrin were analyzed together in one model. Similarly, all 13 inflammatory cytokines were analyzed together in another model, and white blood cell counts were analyzed together in a third model. However, because cytokines are rarely released alone and exhibit a large degree of redundancy and pleiotropy (Ozaki & Leonard, 2002), there was an issue of multicollinearity in the cytokine model. To avoid this issue, we performed principal component analysis (PCA) followed by principal component regression (PCR) using parallel analysis as the method to choose which components to use in the model. Baseline cytokine concentrations were reduced to one component (eigenvalue of 7.19 accounted for 55.3% of the data variability), which was then regressed onto absolute and relative Hb mass.

Table 4. Inflammatory cytokine data from Aim 2.

		Study 1									Study 2					
	LLOD Range	n analyzed Day 1	n < LLOD Day 1	n CV > 30 Day 1	n analyzed Day 2	n < LLOD Day 2	n CV > 30 Day 2	n analyzed Day 3	n < LLOD Day 3	n CV > 30 Day 3	n analyzed Day 1	n < LLOD Day 1	n CV > 30 Day 1	n analyzed Day 2	n < LLOD Day 2	n CV > 30 Day 2
IL-1β (pg/mL)	0.78 - 5.80	17	1	0	17	3	0	17	0	0	34	3	0	7	0	0
IFN-α2 (pg/mL)	0.32 - 1.63	17	0	0	17	0	0	17	0	0	34	0	0	7	0	0
IFN-γ (pg/mL)	0.22 - 3.59	17	0	0	17	1	0	17	0	0	34	2	0	7	1	0
TNF-α (pg/mL)	0.42 - 3.87	17	0	0	17	0	0	17	0	0	34	3	0	7	1	0
MCP-1 (pg/mL)	0.24 - 7.08	17	0	0	17	0	1	17	0	0	34	0	0	7	0	0
IL-6 (pg/mL)	0.22 - 4.43	17	0	0	17	1	0	17	0	0	34	2	0	7	0	0
IL-8 (pg/mL)	0.29 - 23.20	17	0	0	17	0	0	17	0	0	34	2	0	7	0	0
IL-10 (pg/mL)	0.27 - 4.06	17	1	0	17	2	0	17	1	0	34	0	0	7	0	0
IL-12p70 (pg/mL)	0.44 - 2.91	17	2	0	17	4	0	17	1	0	34	2	0	7	0	0
IL-17A (pg/mL)	0.06 - 0.77	17	1	0	17	3	0	17	1	0	34	2	0	7	1	0
IL-18 (pg/mL)	0.79 - 11.22	17	0	0	17	0	0	17	0	0	34	0	1	7	0	0
IL-23 (pg/mL)	0.82 - 10.41	17	0	0	17	0	0	17	0	0	34	3	0	7	1	0
IL-33 (pg/mL)	2.44 - 98.13	17	0	0	17	0	0	17	0	0	34	1	0	7	0	0

Data were collected from three studies, each of which involved 1-3 days in which cytokine concentrations were measured. The number of samples analyzed on each study day are presented ("n analyzed"), as well as the number of samples that were below limits of detection ("n < LLOD") on each study day and for each analyte. The total number of analyzed samples and the mean ± standard deviation (SD) for each analyte are also presented. The total number of analyzed samples does not equal the sum of samples analyzed on each study day because some participants participated in multiple studies, and cytokine concentrations were averaged for these participants. Table continued on following page.

Table 4 continued.

		Study 3			Total	
	LLOD Range	n analyzed Day 1	n < LLOD Day 1	n CV > 30 Day 1	n included	Mean ± SD
IL-1β (pg/mL)	0.78 - 5.80	42	8	0	85	26.21 ± 34.73
IFN-α2 (pg/mL)	0.32 - 1.63	42	1	0	85	35.04 ± 26.25
IFN-γ (pg/mL)	0.22 - 3.59	42	0	0	85	30.62 ± 37.46
TNF-α (pg/mL)	0.42 - 3.87	42	0	0	85	92.83 ± 130.09
MCP-1 (pg/mL)	0.24 - 7.08	42	0	0	85	736.68 ± 255.30
IL-6 (pg/mL)	0.22 - 4.43	42	3	0	85	29.47 ± 29.33
IL-8 (pg/mL)	0.29 - 23.20	42	3	0	85	138.48 ± 109.18
IL-10 (pg/mL)	0.27 - 4.06	42	3	0	85	39.52 ± 52.29
IL-12p70 (pg/mL)	0.44 - 2.91	42	9	0	85	22.22 ± 27.09
IL-17A (pg/mL)	0.06 - 0.77	42	5	0	85	6.08 ± 4.99
IL-18 (pg/mL)	0.79 - 11.22	42	0	0	85	591.44 ± 232.85
IL-23 (pg/mL)	0.82 - 10.41	42	2	0	85	54.32 ± 86.00
IL-33 (pg/mL)	2.44 - 98.13	42	2	0	85	608.29 ± 481.48

RESULTS

Participant characterization

There were 39 females and 51 males that completed the present study. Out of the 39 females, 21 did not have a PFO (PFO-) and 12 had a PFO (PFO+). Out of the 51 males, 30 did not have a PFO (PFO-) and 18 had a PFO (PFO+). There were 6 females and 3 males that did not have PFO screening performed due to either participating only in study 1 in which PFO screening was not part of the study protocol or withdrawing from the study prior to PFO screening. Within each sex, the percentage of PFO+ participants (36.4% of females and 37.5% of males) is representative of the PFO prevalence within the population (Hagen *et al.*, 1984; Marriott *et al.*, 2013; Elliott *et al.*, 2013). All participants, regardless of sex and the presence or absence of a PFO, were approximately 24-26 years old, and there were no differences in

participant age as a result of sex or the presence or absence of a PFO (Table 5). There was an effect of sex but not PFO on height and weight, as expected (Table 5).

Relationships between immune variables & Hb mass

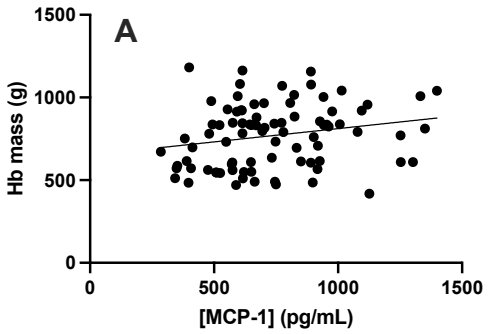
There was a positive linear relationship between MCP-1 and absolute Hb mass (Fig 11A; Table 6). Conversely, there were negative linear relationships between IFN-α2 and neutrophil counts with absolute Hb mass (Fig 11B & C; Table 6). Additionally, neutrophil counts had a negative linear relationship with relative Hb mass (Fig 11D; Table 6). However, although the slopes of the relationships between these immune variables and Hb mass were significantly non-zero, the R^2 of these relationships were all less than 0.10 (Fig 11; Table 6).

Together, white blood cell counts (neutrophils, monocytes, lymphocytes, eosinophils, and basophils) did not predict absolute ($p = 0.3144$; $R^2 = 0.1172$) or relative ($p = 0.1875$; $R^2 = 0.1457$) Hb mass. Similarly, all cytokines together did not predict absolute ($p = 0.0646$; $R^2 = 0.0406$) or relative ($p = 0.2906$; $R^2 = 0.0134$) Hb mass.

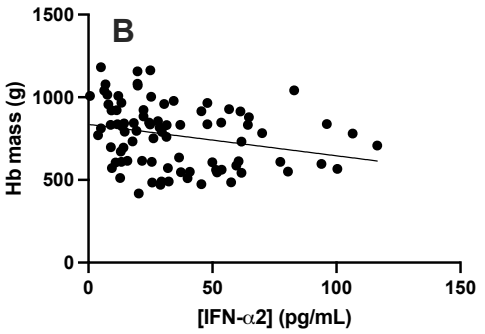
Table 5. Anthropometrics, Hb mass, & blood values in females and males with and without a PFO.

		Females		Males		ANOVA results		
		PFO-	PFO+	PFO-	PFO+	Sex	PFO	Interaction
Anthropometrics	Age (years)	25 ± 5.4	26 ± 7.2	25 ± 5.4	24 ± 4.8	0.6282	0.9216	0.4857
	Height (cm)	163.6 ± 6.4	164.5 ± 5.0	179.9 ± 6.5	180.3 ± 4.6	< 0.0001 *	0.6679	0.8622
	Weight (kg)	61.0 ± 9.3	69.5 ± 12.5	78.6 ± 10.1	78.4 ± 9.4	< 0.0001 *	0.0869	0.0743
Hb mass	Absolute (g)	571.3 ± 78.1	585.3 ± 61.0	890.8 ± 134.3	923.4 ± 105.4	< 0.0001 *	0.3547	0.7109
	Relative (g/kg)	9.5 ± 1.3	8.6 ± 1.4	11.4 ± 1.2	11.9 ± 1.4	< 0.0001 *	0.5903	0.0302 *
Iron status	Iron (mcg/dL)	121.6 ± 37.7	111.8 ± 42.2	113.4 ± 36.9	128.3 ± 49.9	0.6723	0.7901	0.2301
	Ferritin (ng/mL)	28.4 ± 15.7	27.3 ± 13.5	98.8 ± 63.9	60.0 ± 24.7	< 0.0001 *	0.0456 *	0.0596
	Transferrin (mg/dL)	295.3 ± 51.4	296.1 ± 38.6	264.5 ± 42.1	270.7 ± 26.9	0.0051 *	0.7209	0.779
Cytokines	IL-1β (pg/mL)	27.2 ± 25.2	33.6 ± 33.0	24.4 ± 46.5	27.1 ± 27.1	0.6023	0.6053	0.8372
	IFN-α2 (pg/mL)	37.8 ± 19.5	45.5 ± 32.3	32.0 ± 29.1	34.7 ± 28.2	0.2132	0.4399	0.7114
	IFN-γ (pg/mL)	32.2 ± 27.7	30.2 ± 33.9	28.1 ± 46.7	36.2 ± 39.6	0.9216	0.7476	0.6015
	TNF-α (pg/mL)	76.1 ± 69.2	147.2 ± 211.6	105.9 ± 167.0	71.3 ± 49.4	0.4867	0.5815	0.1130
	MCP-1 (pg/mL)	638.5 ± 243.5	659.7 ± 294.2	862.8 ± 245.9	715.0 ± 201.5	0.0209 *	0.2885	0.1578
	IL-6 (pg/mL)	29.4 ± 17.2	35.1 ± 30.3	32.9 ± 42.3	21.3 ± 12.7	0.4886	0.6929	0.2450
	IL-8 (pg/mL)	142.7 ± 78.2	157.4 ± 76.4	146.6 ± 155.2	130.9 ± 72.5	0.6852	0.9839	0.5861
	IL-10 (pg/mL)	38.7 ± 39.6	46.1 ± 52.8	49.7 ± 73.4	26.0 ± 20.6	0.7358	0.5401	0.2457
	IL-12p70 (pg/mL)	22.6 ± 16.7	24.0 ± 18.5	26.9 ± 40.6	16.5 ± 12.3	0.8131	0.5119	0.3938
	IL-17A (pg/mL)	6.5 ± 3.6	5.4 ± 3.1	6.4 ± 6.7	6.4 ± 4.8	0.7245	0.6734	0.6536
	IL-18 (pg/mL)	570.6 ± 186.4	707.2 ± 433.2	598.9 ± 225.8	528.0 ± 140.5	0.1975	0.5723	0.0777
	IL-23 (pg/mL)	38.8 ± 44.1	59.5 ± 55.8	57.3 ± 91.7	74.4 ± 131.2	0.4506	0.3936	0.9339
	IL-33 (pg/mL)	776.2 ± 427.2	623.7 ± 379.8	542.0 ± 500.8	621.3 ± 588.5	0.3285	0.7618	0.3385
WBC counts	Neutrophils (cells/μL)	4569 ± 2239	5006 ± 1707	3520 ± 985	2903 ± 1020	0.0015 *	0.8481	0.2651
	Lymphocytes (cells/μL)	2134 ± 585	1891 ± 126	1923 ± 552	1842 ± 441	0.4640	0.3615	0.6485
	Monocytes (cells/μL)	557 ± 235	546 ± 169	514 ± 150	498 ± 102	0.4108	0.8058	0.9669
	Eosinophils (cells/μL)	147 ± 174	163 ± 89	145 ± 147	159 ± 155	0.9604	0.7678	0.9907
	Basophils (cells/μL)	43 ± 18	45 ± 17	47 ± 23	36 ± 15	0.7336	0.5366	0.3697

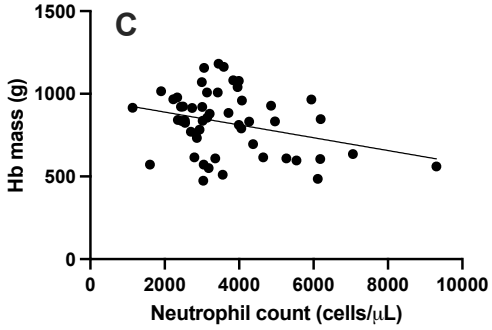
Means ± SDs are presented for all variables for PFO- females, PFO+ females, PFO- males, and PFO+ males. The p-values of the ANOVA results are also presented. The "Sex" column presents the p-values for the main effect of biological sex, the "PFO" column presents the p-values for the main effect of a PFO, and the "Interaction" column presents the p-value of the interactions between PFO and biological sex. Asterisks denote p-values < 0.05. WBC = white blood cell.



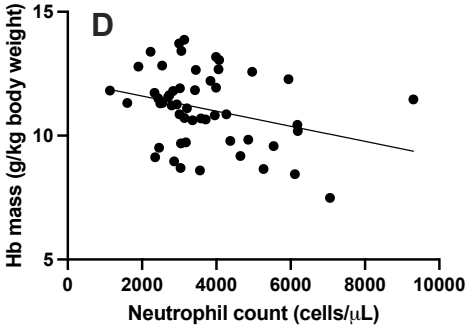
Abs. Hb mass = 0.1605(MCP-1) + 651.2
R^2 = 0.0459
p = 0.0490



Abs. Hb mass = -1.898(IFN-α2) + 836.0
R^2 = 0.0678
p = 0.0161



Abs. Hb mass = -0.03874(Neutrophils) + 966.9
R^2 = 0.0944
p = 0.0268



Rel. Hb mass = -0.0003048(Neutrophils) + 12.21
R^2 = 0.0894
p = 0.0313

Figure 11. Linear relationships between immune variables & Hb mass. Each point represents an individual participant, and lines are the simple linear regressions. A) the relationship between MCP-1 concentration and absolute Hb mass; B) the relationship between IFN-α2 concentration and absolute Hb mass; C) the relationship between neutrophil counts and absolute Hb mass; and D) the relationship between neutrophil counts and relative Hb mass.

Table 6. Hb mass regressions data table.

	Absolute Hb mass			Relative Hb mass		
	Slope	R^2	p-value	Slope	R^2	p-value
Iron (mcg/dL)	0.5778	0.0151	0.2492	0.0072	0.0250	0.1370
Ferritin (ng/mL)	1.6670	0.1893	<0.0001 *	0.0124	0.1105	0.0014 *
Transferrin (mg/dL)	-2.0270	0.2384	<0.0001 *	-0.0173	0.1840	<0.0001 *
IL-1β (pg/mL)	-1.1060	0.0403	0.0655	-0.0056	0.0110	0.3406
IFN-α2 (pg/mL)	-1.8980	0.0678	0.0161 *	-0.0101	0.0203	0.1931
IFN-γ (pg/mL)	-0.7066	0.0191	0.2069	-0.0021	0.0017	0.7039
TNF-α (pg/mL)	-0.2270	0.0238	0.1585	-0.0011	0.0056	0.4963
MCP-1 (pg/mL)	0.1605	0.0459	0.0490 *	0.0011	0.0243	0.1541
IL-6 (pg/mL)	-1.3110	0.0404	0.0652	-0.0089	0.0196	0.2011
IL-8 (pg/mL)	-0.2265	0.0167	0.2386	-0.0013	0.0055	0.4490
IL-10 (pg/mL)	-0.5575	0.0232	0.1639	-0.0020	0.0032	0.6064
IL-12p70 (pg/mL)	-1.2900	0.0333	0.0944	-0.0073	0.0111	0.3369
IL-17A (pg/mL)	-4.6870	0.0149	0.2650	-0.0426	0.0130	0.2989
IL-18 (pg/mL)	-0.0718	0.0076	0.4268	-0.0007	0.0074	0.4334
IL-23 (pg/mL)	0.0471	0.0004	0.8474	0.0000	0.0000	0.9859
IL-33 (pg/mL)	-0.0772	0.0377	0.0750	-0.0005	0.0194	0.2042
Neutrophils (cells/mL)	-0.0387	0.0944	0.0268 *	-0.0003	0.0894	0.0313 *
Lymphocytes (cells/mL)	-0.0253	0.0047	0.6278	0.0000	0.0011	0.8140
Monocytes (cells/mL)	0.1410	0.0140	0.4032	-0.0003	0.0013	0.8033
Eosinophils (cells/mL)	-0.0902	0.0050	0.6179	-0.0019	0.0349	0.1849
Basophils (cells/mL)	-0.3353	0.0013	0.8012	-0.0143	0.0354	0.1814

Slopes, R^2 values, and p-values are presented for the regressions between each of the iron and immune variable predictors and absolute and relative Hb mass. Asterisks denote p-values < 0.05.

Relationships between iron variables & Hb mass

There was a positive linear relationship between ferritin and absolute Hb mass and a negative linear relationship between transferrin and absolute Hb mass (Fig 12A & B; Table 6). A similar pattern was observed in the relationships with relative Hb mass – there was a positive linear relationship between ferritin and relative Hb mass and a negative linear relationship

between transferrin and relative Hb mass (Fig 12C & D; Table 6). The R^2 of these relationships ranged from approximately 0.11 to 0.24. Iron, ferritin, and transferrin together predicted absolute ($p < 0.0001$; $R^2 = 0.3130$) and relative ($p < 0.0001$; $R^2 = 0.2313$) Hb mass.

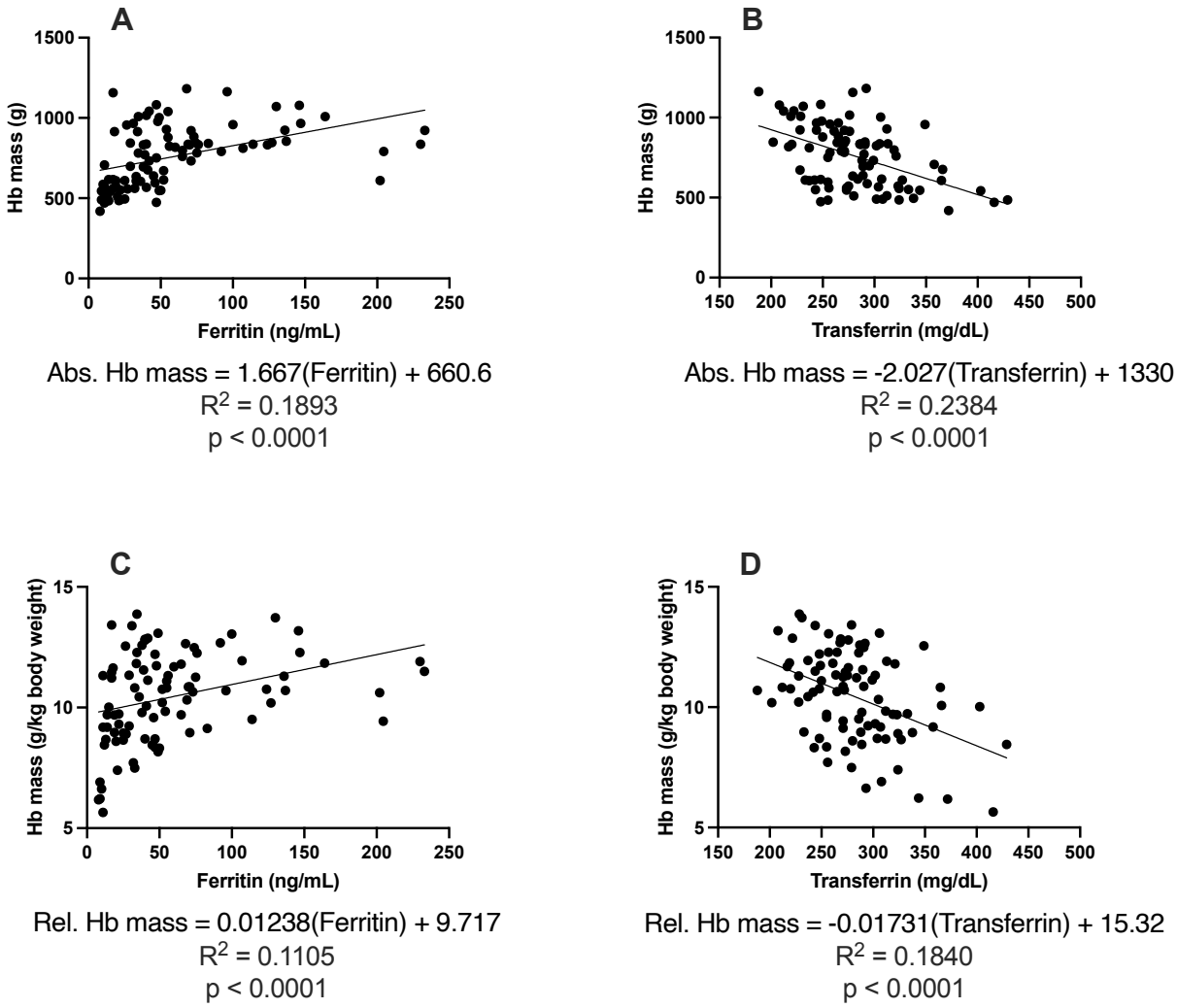


Figure 12. Linear relationships between iron variables & Hb mass. Each point represents an individual participant, and lines are the simple linear regressions. A) the relationship between ferritin and absolute Hb mass; B) the relationship between transferrin and

absolute Hb mass; C) the relationship between ferritin and relative Hb mass; D) the relationship between transferrin and relative Hb mass.

Effect of PFO and sex on iron & immune variables

The only variable affected by the presence or absence of a PFO was ferritin, with PFO+ males exhibiting lower ferritin concentrations compared to PFO- males (Fig 13; Table 5). When the effect of sex was not considered and PFO- and PFO+ participants were compared, there were no differences in ferritin between PFO- and PFO+ participants ($p = 0.0537$). Additionally, females were found to have lower ferritin, higher transferrin, and lower MCP-1 concentrations compared to males, but there were no interactions between sex and PFO on any immune variable or iron variable (Table 5).

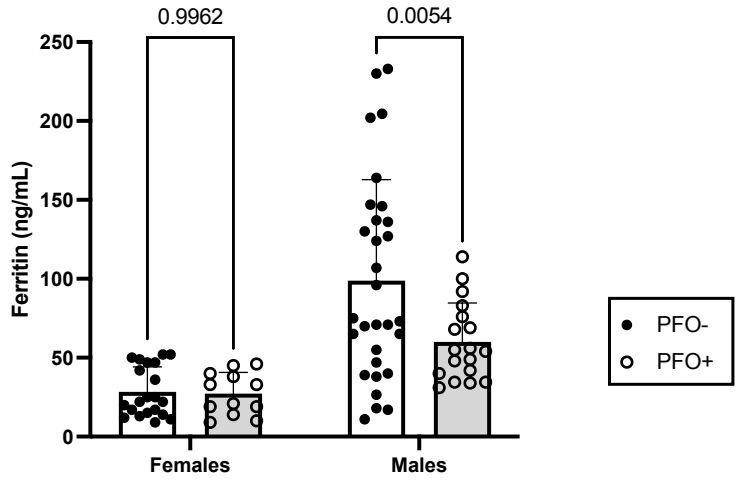


Figure 13. PFO, sex and ferritin. Ferritin concentration was compared between PFO- (closed circles, white bars) to PFO+ (open circles, gray bars) participants within each sex. Data are presented as mean ± SD. Brackets above the bars indicate p-values of pairwise comparisons.

Effect of PFO and sex on Hb mass

Females had lower absolute (Fig 14A; Table 5) and relative (Fig 14B; Table 5) Hb mass compared to males, as expected. However, there were no effects of a PFO on absolute (Fig 14A; Table 5) or relative (Fig 14B; Table 5) Hb mass. Interestingly, however, there was an interaction between sex and PFO on relative Hb mass only, but pairwise comparisons indicated there were no significant differences in relative Hb mass between PFO- and PFO+ participants within each sex (Fig 14B).

Presence of a PFO on the relationships between iron & immune variables with Hb mass

The presence of a PFO affected the slope of the relationship between blood iron and absolute Hb mass as well as the slopes of the relationships between IL-6 and monocyte counts with relative Hb mass (Fig 15; Table 7). The slopes of these relationships were significantly different between those with and without a PFO (Fig 15; Table 7). The slope of the relationship between iron concentration and absolute Hb mass was only non-zero in those with a PFO, while there was no relationship between iron and absolute Hb mass in those without a PFO. A similar pattern was observed with the relationships between IL-6 and monocyte counts with relative Hb mass in that the slope of the relationships were only non-zero in those with a PFO (Fig 15; Table 7).

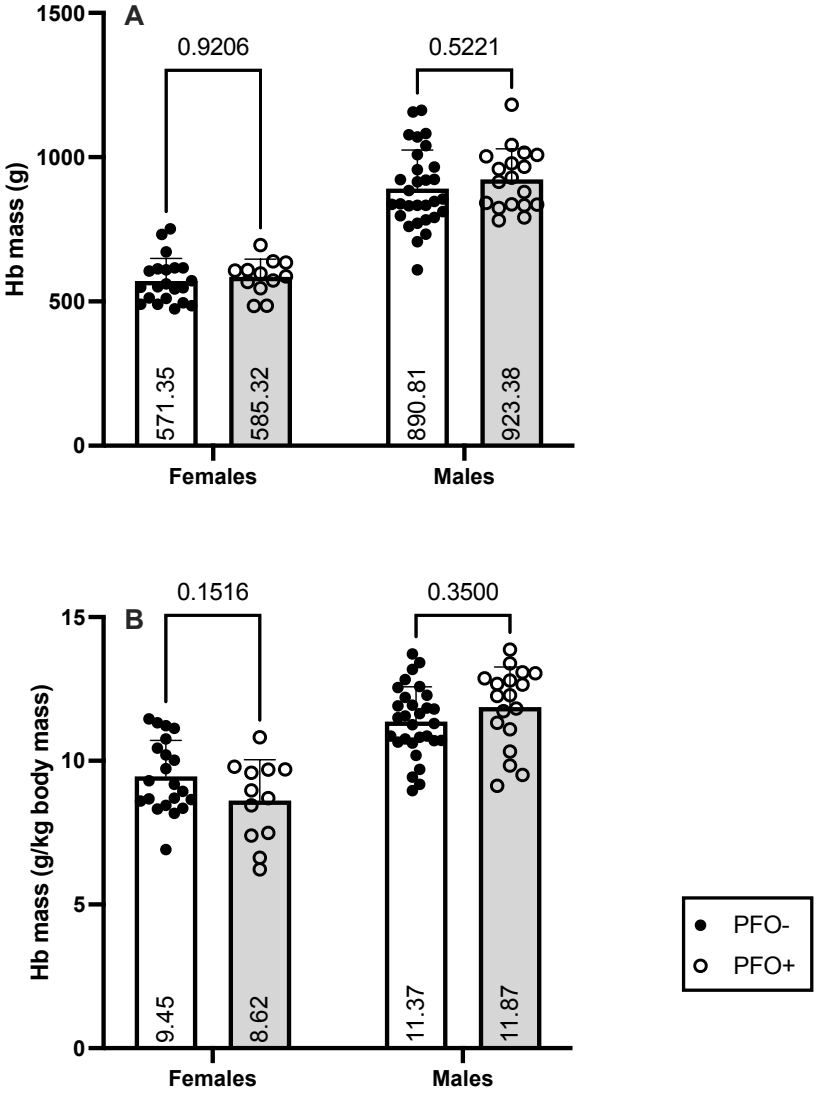


Figure 14. PFO, sex and Hb mass. A) Comparing absolute Hb mass (g) between PFO- (closed circles, white bars) to PFO+ (open circles, gray bars) participants within each sex. B) Comparing relative Hb mass (g/kg) between PFO- (closed circles, white bars) to PFO+ (open circles, gray bars) participants within each sex. Data are presented as mean ± SD. Brackets above the bars indicate p-values of pairwise comparisons. Values within the bars are the group means.

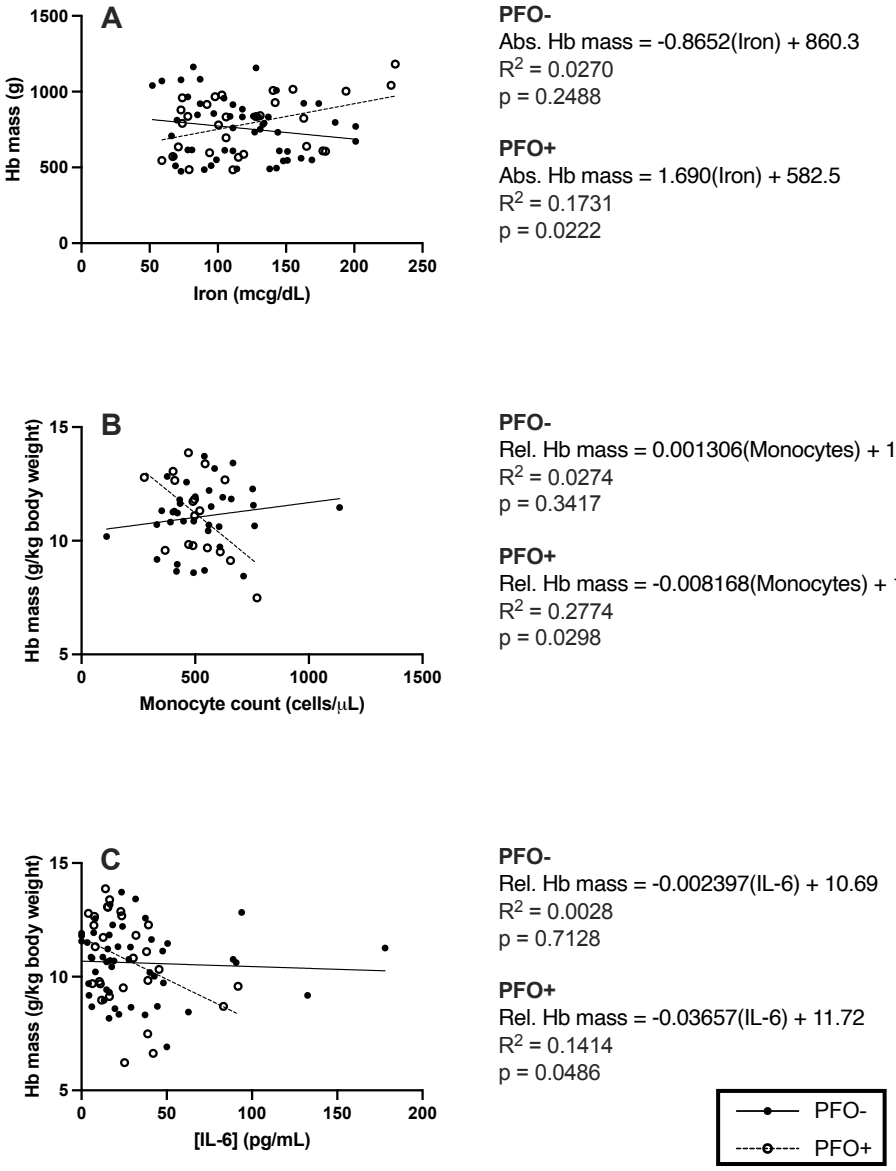


Figure 15. Linear relationships associated with the presence/absence of a PFO. Each point represents an individual participant, and lines are the simple linear regressions. PFO- participants are presented in closed circles with a solid line, and PFO+ participants are presented in open circles with a dashed line. A) the relationship between iron and absolute Hb mass in PFO- and PFO+ participants; B) the relationship between monocyte count and relative Hb mass in PFO- and PFO+ participants; and C) the relationship between IL-6 concentration and relative Hb mass in PFO- and PFO+ participants.

Table 7. Regressions in those with and without a PFO.

	Absolute Hb mass								Relative Hb mass							
	PFO-			PFO+			Slopes different?	Intercepts different?	PFO-			PFO+			Slopes different?	Intercepts different?
	Slope	R^2	p-value	Slope	R^2	p-value	p-value	p-value	Slope	R^2	p-value	Slope	R^2	p-value	p-value	p-value
Iron (mcg/dL)	-0.8652	0.0270	0.2488	1.6900	0.1731	0.0222 *	0.0157 *	NA	-0.0051	0.0152	0.3887	0.0138	0.0919	0.1035	0.0536	0.9475
Ferritin (ng/mL)	1.4530	0.2045	0.0009 *	3.5260	0.2370	0.0064 *	0.1121	0.1095	0.0082	0.1050	0.0204 *	0.0303	0.1404	0.0413 *	0.0824	0.5686
Transferrin (mg/dL)	-1.9560	0.2332	0.0003 *	-2.2220	0.1563	0.0306 *	0.8068	0.3667	-0.0101	0.0996	0.0241	-0.0285	0.2059	0.0118	0.0782	0.9085
IL-1β (pg/mL)	-1.1380	0.0528	0.1085	-1.2720	0.0392	0.3123	0.9257	0.3197	-0.0067	0.0288	0.2386	-0.0023	0.0011	0.8696	0.7444	0.6800
IFN-α2 (pg/mL)	-2.3920	0.1002	0.0251 *	-1.6550	0.0696	0.1749	0.6421	0.2678	-0.0082	0.0188	0.3426	-0.0158	0.0510	0.2477	0.6138	0.6327
IFN-γ (pg/mL)	-0.8836	0.0332	0.2054	-0.4551	0.0083	0.6448	0.7225	0.3409	-0.0034	0.0076	0.5482	0.0007	0.0001	0.9502	0.7171	0.7056
TNF-α (pg/mL)	-0.1414	0.0099	0.4913	-0.4431	0.1015	0.0985	0.3742	0.3633	-0.0001	0.0001	0.9387	-0.0034	0.0509	0.2485	0.2827	0.7099
MCP-1 (pg/mL)	0.2318	0.1021	0.0237 *	0.1067	0.0182	0.4938	0.4944	0.2186	0.0014	0.0595	0.0878	0.0011	0.0169	0.5094	0.8778	0.5419
IL-6 (pg/mL)	-0.8624	0.0234	0.2890	-3.2210	0.1365	0.0530	0.2096	0.4611	-0.0024	0.0028	0.7128	-0.0366	0.1414	0.0486 *	0.0471 *	NA
IL-8 (pg/mL)	-0.2312	0.0237	0.2854	-0.2749	0.0119	0.5809	0.9361	0.3924	-0.0014	0.0133	0.4258	-0.0006	0.0004	0.9187	0.8723	0.7314
IL-10 (pg/mL)	-0.4205	0.0181	0.3523	-1.4670	0.0806	0.1431	0.3467	0.4700	-0.0009	0.0013	0.8014	-0.0114	0.0388	0.3152	0.3107	0.7782
IL-12p70 (pg/mL)	-1.0870	0.0344	0.1974	-3.6080	0.0843	0.1339	0.3277	0.4813	-0.0046	0.0098	0.4951	-0.0393	0.0805	0.1436	0.1444	0.8109
IL-17A (pg/mL)	-6.8120	0.0391	0.1689	0.5853	0.0002	0.9465	0.4604	0.4026	-0.0606	0.0487	0.1234	-0.0073	0.0002	0.9403	0.5614	0.7522
IL-18 (pg/mL)	0.1004	0.0118	0.4525	-0.1787	0.0765	0.1542	0.1287	0.3805	0.0006	0.0071	0.5612	-0.0010	0.0197	0.4765	0.3356	0.7197
IL-23 (pg/mL)	-0.0477	0.0004	0.8969	0.0401	0.0006	0.9051	0.8602	0.3857	-0.0017	0.0074	0.5519	0.0008	0.0017	0.8350	0.5805	0.7091
IL-33 (pg/mL)	-0.1206	0.0903	0.0339 *	-0.0327	0.0083	0.6454	0.3277	0.3853	-0.0007	0.0450	0.1391	-0.0006	0.0236	0.4352	0.9411	0.7330
Neutrophils (cells/mL)	-0.0293	0.0491	0.2010	-0.0543	0.2384	0.0467 *	0.5070	0.4891	-0.0002	0.0317	0.3061	-0.0006	0.2541	0.0391 *	0.1341	0.9171
Lymphocytes (cells/mL)	0.0021	0.0000	0.9735	-0.1167	0.0758	0.2848	0.3866	0.3724	0.0003	0.0132	0.5111	-0.0007	0.0200	0.5878	0.3982	0.8331
Monocytes (cells/mL)	-0.0346	0.0009	0.8613	-0.6058	0.1863	0.0836	0.2015	0.3645	0.0013	0.0274	0.3417	-0.0082	0.2774	0.0298 *	0.0079 *	NA
Eosinophils (cells/mL)	-0.0390	0.0009	0.8642	-0.2496	0.0455	0.4112	0.6030	0.3322	-0.0005	0.0029	0.7597	-0.0056	0.1887	0.0814	0.1104	0.8040
Basophils (cells/mL)	0.2985	0.0011	0.8514	-1.9530	0.0326	0.4878	0.5219	0.3615	-0.0056	0.0078	0.6135	-0.0529	0.1960	0.0751	0.0890	0.9601

Slopes, R^2 values, and p-values are presented for the regressions between each of the predictors and absolute and relative Hb mass in PFO- and PFO+ participants. The slopes and intercepts of the linear regressions between PFO- and PFO+ were compared, and p-values are presented for these comparisons in the columns titled "Slopes different?" and "Intercepts different?". Asterisks denote p-values < 0.05.

DISCUSSION

The purpose of this study was to examine the role of iron status parameters, immune system components, and a PFO in modulating sea level Hb mass to help explain the large degree of inter-individual variability in this measurement. We hypothesized that low iron availability, high cytokine concentrations, and high white blood cell counts would correlate with low Hb mass, and that the presence of a PFO would result in lower Hb mass primarily by reducing iron availability. In support of our hypothesis, we found baseline blood concentrations of certain immune system and iron variables had linear relationships with absolute and relative Hb mass. Contrary to our hypothesis, we found the presence of a PFO did not result in lower Hb mass, and we also show that the presence of a PFO did not result in higher concentrations of inflammatory cytokines or white blood cell counts. However, the presence of a PFO did result in reduced ferritin in males, and the presence of a PFO altered the relationships between some immune system components and iron parameters with Hb mass.

Immune system & Hb mass

We found only some of the immune variables examined in the present study had significant relationships with Hb mass irrespective of the presence or absence of a PFO, primarily IFN-α2, MCP-1, and neutrophil counts. The negative linear relationship between IFN-α2 and Hb mass is expected, as IFN-α2 has been extensively studied and has been shown to reduce Hb mass via numerous pathways, including suppressing colony formation and proliferation of erythroid precursor cells as well as causing apoptosis of erythroid cells (Broxmeyer *et al.*, 1983; Raefsky *et al.*, 1985; Lai *et al.*, 1995; Tarumi *et al.*, 1995).

However, in contrast to IFN-α2, no studies to date have shown a modulatory role of MCP-1 on Hb mass. Interestingly, we found the relationship between MCP-1 and Hb mass was a

positive linear relationship, which is the opposite of the hypothesized negative relationship given MCP-1 is typically a pro-inflammatory chemokine. The reasons for this positive linear relationship are unknown. It could be that MCP-1 stimulates red blood cell formation or survival via an unknown mechanism, or this result could simply be the result of an associative relationship and not represent an active modulatory role of MCP-1 on red blood cells.

Interestingly, MCP-1 is responsible for recruiting monocytes, so it is unexpected that we did not observe a linear relationship between monocytes and Hb mass when there was a linear relationship between MCP-1 and Hb mass. Additionally, monocytes can differentiate into macrophages, which are responsible for recycling old red blood cells, making it more intriguing monocytes did not appear to have a significant relationship with Hb mass. The only immune cells found to have a linear relationship with Hb mass were neutrophils, and similar to MCP-1, there are no studies to suggest the potential mechanism by which neutrophils could modulate Hb mass. However, this may be a result of the present study showing only an association between these variables rather than a direct regulatory role. More research should be done to determine whether MCP-1 and neutrophils actively regulate Hb mass by inhibiting these specific immune system components and/or treating participants with a non-specific anti-inflammatory drug to determine a more causal role of these immune system components.

Interestingly, we found most immune system components that we examined did not have a linear relationship with either absolute or relative Hb mass. This seemingly contradicts previous work in cell culture and animal models which demonstrated multiple immune cells and inflammatory cytokines can negatively affect red blood cell formation and survival (Mamus *et al.*, 1985; Raefsky *et al.*, 1985; Schooley *et al.*, 1987; Wang *et al.*, 1995; Tarumi *et al.*, 1995; Xiao *et al.*, 2002; Felli *et al.*, 2005; Libregts *et al.*, 2011; Prince *et al.*, 2012; McCranor *et al.*,

2014; Pietras *et al.*, 2016; Etzrodt *et al.*, 2019; Lu *et al.*, 2020; Swann *et al.*, 2020). However, cytokines are well known to act differently depending on the microenvironment and co-release of other cytokines, so the seemingly contradictory results may be partially due to the experimental models used. For example, IL-6 can both stimulate EPO (Faquin *et al.*, 1992; Vannucchi *et al.*, 1994) and inhibit Hb synthesis (McCranor *et al.*, 2014) in cell culture. Similarly, IL-17 can promote the development of some erythroid precursor cells but inhibit others, and whether it acts in an inhibitor or stimulatory fashion is dependent on whether it's acting in the spleen or bone marrow (Krstic *et al.*, 2012; Mojsilović *et al.*, 2015). Therefore, the results of the present study may be a result of more complicated immune relationships that do not exist in a reductionist cell culture model or animal model, which likely are not representative of human physiology.

Despite this, there are data showing humans with anemia of chronic disease have higher circulating concentrations of inflammatory cytokines (Keithi-Reddy *et al.*, 2008). It could therefore be expected that we would see lower Hb mass in our participants with higher concentrations of cytokines. However, our study utilized a healthy human model. It is possible that in otherwise healthy humans, concentrations of inflammatory cytokines are simply not high enough to disrupt red blood cell regulation or are met with a compensatory mechanism to maintain Hb mass. In our participants that exhibited higher concentrations of cytokines, it could be that the concentrations of cytokines that we quantified were only elevated acutely at the time of our measurement. If a chronic systemic low-grade inflammation was induced in healthy humans, it is possible that red blood cell dysregulation would be evident.

One additional explanation for not observing significant relationships between inflammation and Hb mass may be the result of only studying baseline sea level conditions. It

may be that inflammatory cytokines and/or immune cells only play a regulatory role in Hb mass in healthy humans in situations where red blood cell synthesis increases, such as during chronic altitude exposure or with exercise training. Lastly, an important point is the relationships between the immune system measures and Hb mass should be interpreted cautiously because the R^2 of these regressions range from approximately 0.045 to 0.094, indicating each of these variables on their own do not predict a very large percent of the total Hb mass. Additionally, when analyzed together in a multiple linear regression or principal component regression, white blood cells and cytokines do not significantly predict absolute or relative Hb mass.

Iron & Hb mass

This study showed ferritin and transferrin concentrations had linear relationships with absolute Hb mass. Interestingly, when considering all participants regardless of the presence or absence of a PFO, total iron concentration did not have a significant relationship with Hb mass. Therefore, iron storage and transport may be more important in the formation of red blood cells than total blood iron. In support of this, one study which used chronic COi to increase Hb mass showed the increase in Hb mass occurred alongside decreased ferritin (Schmidt *et al.*, 2020). Similarly, increases in Hb mass with chronic altitude exposure have been shown to occur alongside decreased ferritin concentration (Govus *et al.*, 2015; Koivisto-Mørk *et al.*, 2021), and, upon return to sea level, post-altitude sojourn ferritin concentrations increase while Hb mass decreases (Garvican *et al.*, 2012). Additionally, Goodrich *et al.* (2020) found ferritin had a significant relationship with Hb mass normalized to lean body mass in their female participants. However, no studies to date have quantified the relationship between transferrin and Hb mass that we report in the present study. Therefore, for future studies examining iron availability and red blood cell regulation, considering transferrin concentration may be of particular importance.

PFO & Hb mass

This study showed the presence of a PFO did not affect absolute Hb mass. However, there was an interaction between biological sex and a PFO on relative Hb mass. The most likely explanation for the significant interaction on relative Hb mass but not absolute Hb mass are differences in participant weight. The mean relative Hb mass of PFO+ females was approximately 0.9 g/kg lower than the mean relative Hb mass of PFO- females, whereas the mean relative Hb mass of PFO+ males was approximately 0.5 g/kg higher than the mean relative Hb mass of PFO- males. However, while the average weights of PFO- and PFO+ males were nearly identical, the PFO+ females included in this study on average weighed approximately 8.5 kg more than PFO- females. Higher body weights would lead to lower relative Hb mass in PFO+ females when absolute Hb mass is similar, so this is the most likely explanation for why we observe a significant PFO and sex interaction in relative Hb mass but not in absolute Hb mass. To further explore if this is a plausible explanation, we estimated lean body mass using the Boer equation (Boer, 1984) and determined that there was an effect of sex ($p < 0.0001$) but not PFO ($p = 0.8053$) on Hb mass normalized to estimated lean body mass. PFO- females had approximately 12.9 g of Hb/kg of lean body mass while PFO+ females had approximately 12.5 g of Hb/kg of lean body mass. This likely indicates that the PFO+ females in the present study had more fat mass but not more lean mass, resulting in the observed PFO and sex interaction on Hb mass relative to total body mass.

PFO & iron

An interesting result from this study was males with a PFO had significantly lower ferritin than males without a PFO. Importantly, both the presence of a PFO and iron deficiency are associated with increased incidence of stroke, and it is possible higher incidence of iron

deficiency in those with a PFO partially contributes to increased stroke risk (Shovlin, 2014; Topiwala *et al.*, 2021). We are the first to show a potential link between the presence of a PFO and iron deficiency and that the presence of a PFO alters iron availability in young, healthy males. However, lower ferritin was only evident within the males. When PFO- participants were compared to PFO+ participants regardless of sex, there was no effect of a PFO. Why this was only seen in males is unknown but warrants future investigation to determine if this is a repeatable finding.

However, despite the presence of a PFO resulting in lower ferritin in males, the presence of a PFO did not result in a significantly different slope or intercept of the linear relationship between ferritin and Hb mass, indicating the presence of a PFO does not affect the relationship between these variables. Additionally, it appears contradictory that males with a PFO had lower ferritin, and ferritin has a significant relationship with Hb mass, yet males with a PFO did not have lower Hb mass compared to males without a PFO. The most likely explanation is ferritin is not the only modulatory factor of Hb mass. The R^2 of the relationship between ferritin and absolute Hb mass was approximately 0.19. Because ferritin only predicts approximately 19% of absolute Hb mass, a significantly lower ferritin concentration in PFO+ males does not necessarily have to translate to a lower Hb mass in PFO+ males. There are no studies which have examined iron regulation in those with and without a PFO and how that might affect Hb mass, so whether this is a repeatable finding or simply due to the participants included in this study is unknown.

An additional unexpected finding in the present study is the presence of a PFO resulted in a significantly different slope of the relationship between iron and absolute Hb mass. The reasons for this, and the extent to which this is a physiologically relevant finding, are unknown

and require further investigation. There are data to suggest biological sex can influence the relationships between iron variables and Hb mass (Goodrich *et al.*, 2020), but no studies to date have examined the potential mechanisms to explain why the presence of a PFO alters the relationship between iron and Hb mass.

Effects of sex assigned at birth

While not the primary purpose of our study, we have confirmed females have significantly lower absolute and relative Hb mass compared to males, which is supported by previous work (Falz *et al.*, 2019; Goodrich *et al.*, 2020). In addition, there was an effect of sex on ferritin and transferrin concentrations, as well as MCP-1 concentration and neutrophil counts. These results are expected, as others have found sex differences in iron and immune regulation (Klein & Flanagan, 2016; Falz *et al.*, 2019; Cappellini *et al.*, 2020).

Given the purpose of the study was not to examine the known sex differences in Hb mass, data were not initially included on whether sex assigned at birth affected the relationship between iron and immune variables with Hb mass. However, the slope of the relationship between ferritin and absolute Hb mass was significantly different between males and females. There was a significantly non-zero slope in females only, with an R^2 in females of approximately 0.28 while the R^2 in males was only 0.017. It is interesting to speculate iron stores may be more meaningful in modulating Hb mass in females compared to males. Goodrich *et al.* (2020) found comparable results, showing the relationship between ferritin and Hb mass was only significant in females with low ferritin. It is possible that because ferritin is higher in males than in females, ferritin is not the limiting factor in the formation of Hb in males, which would lead to an insignificant relationship between ferritin and Hb mass in males.

Limitations

Although this study provides novel data quantifying the associations between inflammation, iron, the presence or absence of a PFO, and Hb mass, the present study was not an interventional study. The only way to directly understand the causal effect of inflammation, iron, and the presence or absence of a PFO on Hb mass would be to design a study which alters inflammation and/or iron availability or would be done in participants before and after percutaneous PFO closure. Furthermore, fluctuations in inflammatory cytokine concentrations and iron availability can be transient and change more quickly than Hb mass will change, so examining these associations at a single time point minimizes our ability to determine a more regulatory role of these cytokines. It is possible the concentrations of cytokines and iron parameters we measured on a given day are not representative of the normal range for that participant and do not represent the concentrations which would normally play a more influential role on Hb mass. However, we had a small number of participants complete all three studies. In these participants, there were no data to suggest that fluctuations in Hb mass correlated with fluctuations in iron parameters or immune system components. In other words, the lowest Hb mass quantified within a single participant did not necessarily coincide with the highest inflammation and/or lowest iron availability. Additionally, the data in these participants did not fluctuate dramatically. Even across a span of months, iron and immune parameters as well as Hb mass remained relatively consistent within a participant.

Conclusions & future directions

Overall, our study shows under sea level conditions there are some moderate relationships between immune system components (i.e., white blood cells and cytokines), iron status, and Hb mass. Additionally, the presence of a PFO did not affect Hb mass or any immune

system measures made in this study, but it surprisingly resulted in lower ferritin in PFO+ males. Lastly, the presence of a PFO may be important in modulating the relationship between iron, immune variables, and Hb mass. Importantly, these data provide a basis for future work to more directly explore the mechanisms of interaction between the immune system and Hb mass and concentration. Future studies should focus on interventions that alter iron availability and/or immune system activity and determine how that alters chronic Hb mass regulation. Additionally, studying these parameters pre- and post-percutaneous PFO closure would provide useful insight into the direct role of a PFO on iron availability, immune activity, and Hb mass regulation.

CHAPTER VI

NON-DECOMPRESSION SCUBA DIVING INCREASES INFLAMMATION BUT NOT PULMONARY PRESSURE

This chapter will be submitted to *The Journal of Science and Medicine in Sport* with Joel E. Futral, Dr. Rachel N. Lord, Dr. Otto Barak, Dr. Justin Edward, Dr. Ivan Drvis, Dr. Igor Glavičić, Ivana Miloš, Dr. Željko Dujić, and Dr. Andrew T. Lovering as co-authors. All experimental work was performed either by me independently or by J.E.F., R.N.L., O.B., J.E., and A.T.L. under my direction. The writing is entirely mine. All co-authors provided editorial assistance.

INTRODUCTION

PASP increases in humans following SCUBA diving and remains elevated hours after a dive has occurred (Dujić *et al.*, 2006; Marabotti *et al.*, 2013). Importantly, these studies utilized decompression dive profiles (Dujić *et al.*, 2006; Marabotti *et al.*, 2013), so it is unknown whether PASP increases following non-decompression dives. In addition, the mechanisms responsible for the increase in PASP post SCUBA diving remain unclear. It is unknown whether the elevated PASP post SCUBA diving is a result of increased Q and/or increased TPR from pulmonary vasoconstriction. However, it is hypothesized to be a result of sustained pulmonary vasoconstriction secondary to elevated vasoactive substances that remain in circulation after a dive rather than the effects of exercise, immersion, hyperoxia, and hyperbaria that occur during a dive, although this has not been confirmed.

NO is a potent vasodilator that regulates pulmonary vascular tone in humans during both normoxia (Cooper *et al.*, 1996) and hypoxia (Blitzer *et al.*, 1996; Bailey *et al.*, 2010), and sildenafil, which increases NO bioavailability, has been shown to improve pulmonary

hypertension (Barnes *et al.*, 2019), reduce pulmonary pressure with hypoxia (Carter *et al.*, 2019; Kelly *et al.*, 2022), and reduce pulmonary pressure with immersion pulmonary edema (Moon *et al.*, 2016; Martina *et al.*, 2017). Therefore, post SCUBA diving NO bioavailability may alter pulmonary vascular tone, and sildenafil may be effective at reducing pulmonary pressure post SCUBA diving. Additionally, while not as potent as NO, circulating inflammatory cytokines can also modulate pulmonary vascular tone. For example, high concentrations of IL-1β and TNF-α have been shown to augment hypoxic pulmonary vasoconstriction (Tsai *et al.*, 2004), implicating these cytokines as modulators of acute PASP. Importantly, it has been shown that SCUBA diving alters endothelial function (Brubakk *et al.*, 2005; Culic *et al.*, 2014) and increases immune system activity (Eftedal *et al.*, 2013; Sureda *et al.*, 2014; Žarak *et al.*, 2021; Rocco *et al.*, 2021), so baseline inflammatory cytokine concentrations and/or the increase in circulating cytokine concentrations after a dive may further modulate pulmonary vascular tone.

Lastly, the presence of a PFO may alter PASP regulation post SCUBA diving. No studies have examined the role of a PFO in modulating PASP with hyperoxia, hyperbaria, or SCUBA diving, but there are studies that examine the modulatory role of a PFO on pulmonary pressure during hypoxia. The literature on the effect of a PFO on pulmonary pressure is mixed, with some studies showing no effect of a PFO (Duke *et al.*, 2020; DiMarco *et al.*, 2021), while others show that the presence of a PFO is associated with susceptibility for developing high altitude pulmonary edema, a condition characterized by elevated pulmonary pressure (Allemann *et al.*, 2006). The role of a PFO in modulating PASP post SCUBA diving is unknown.

Therefore, the purpose of this study was to examine whether two different non-decompression dive profiles caused PASP to increase, and whether the increase was due to increased Q and/or increased TPR. In addition, we determined whether NO bioavailability and/or

the presence or absence of a PFO altered PASP post SCUBA diving. Lastly, we examined the effect of SCUBA diving on cytokine concentration and associations between pulmonary pressure and cytokine concentrations. We hypothesized that PASP and TPR would increase post SCUBA diving and that this would be reversible with sildenafil (50 mg, p.o.), that those with the greatest pre and post-dive serum concentrations of pro-inflammatory cytokines would have the greatest increase in PASP and TPR, and that the presence of a PFO would not alter PASP or TPR.

METHODS

Ethical approval

A total of 50 individuals volunteered to participate in this study after being advised both verbally and in writing as to the nature of the experiments. Participants signed an informed consent form to participate in the study approved by the University of Oregon Research Compliance Services and University of Split School of Medicine Ethics Committee (University of Oregon protocol #07302018.031; University of Split School of Medicine protocol #2181-198-03-04-19-0052). All studies were performed in accordance with the standards set forth by the *Declaration of Helsinki,* and this study is registered at clinicaltrials.gov (protocol #07302018.031). All participants held at minimum either a PADI Open Water Diver certification or a CMAS 1 Star certification and were recruited at Big Blue Diving in Croatia. All participants were given the option to complete the informed consent process in Croatian. All data collection occurred in the city of Bol, on the island of Brac, Croatia. Age, sex, and anthropometric data from the 50 participants that completed the study are presented in Table 8.

Screening

All participants performed spirometry including forced vital capacity maneuvers. Pulmonary function testing was done according to societal standards (Miller *et al.*, 2005).

Absolute and Global Lung Initiative (GLI) calculated percent of age and sex predicted values for forced vital capacity, forced expiratory volume in 1 second, forced expiratory volume in 1 second to forced vital capacity ratio, and mid expiratory flow are presented in Table 8.

Table 8. Anthropometrics and comprehensive cardiopulmonary characterization.

		Shallow profile	Deep profile
Anthropometrics	Age	43.3 ± 12.2	43.9 ± 10.3
	Height (cm)	177.3 ± 8.3	178.0 ± 8.6
	Weight (kg)	81.1 ± 15.7	84.2 ± 18.2
	n females, males	6, 20	4, 20
	n PFO+, PFO-	13, 13	7, 13
Spirometry	FVC (L)	5.2 ± 1.0	5.1 ± 0.9
	FVC (% pred)	105.5 ± 9.1	102.0 ± 11.6
	FEV1 (L)	4.1 ± 0.8	4.0 ± 0.7
	FEV1 (% pred)	102.1 ± 12.5	98.7 ± 13.2
	FEV1/FVC	0.78 ± 0.08	0.77 ± 0.07
	FEV1/FVC (% pred)	96.4 ± 10.4	96.4 ± 8.2
	FEF25-75 (L)	3.7 ± 1.1	3.6 ± 1.2
	FEF25-75 (% pred)	100.0 ± 31.2	95.3 ± 29.9
Comprehensive echo	RV wall	5.3 ± 0.9	4.1 ± 1.1
	IVSd	9.3 ± 1.5	9.3 ± 1.4
	LVIDd	46.9 ± 3.9	48.9 ± 5.4
	LVPWd	9.4 ± 1.5	10.1 ± 1.5
	LVIDs	31.3 ± 3.6	28.6 ± 6.5
	LA s	32.0 ± 5.2	34.0 ± 4.0
	PV pk vel	1.0 ± 0.2	1.0 ± 0.2
	LVOT vel	1.1 ± 0.2	1.1 ± 0.2
	AoV vel	1.4 ± 0.4	1.2 ± 0.3
	AoV VTI	26.5 ± 4.0	23.3 ± 4.1
	AVA	3.0 ± 0.6	6.1 ± 1.8
	MV-E vel	0.7 ± 0.2	0.7 ± 0.2
	MV-A vel	0.6 ± 0.2	0.5 ± 0.1
	LAT e'	0.2 ± 0.2	0.2 ± 0.0
	E/e' ratio	5.2 ± 1.7	4.6 ± 0.9
	RVED area	18.1 ± 4.7	25.0 ± 5.0
	RVES area	11.9 ± 3.4	13.3 ± 3.0
	%RVEF	34.0 ± 8.4	46.9 ± 5.5
	LVED vol	106.0 ± 26.3	131.2 ± 25.2
	LVES vol	46.9 ± 11.5	51.6 ± 13.6
	HR at LV vol meas	71.5 ± 13.8	70.6 ± 9.5
	%LVEF	55.0 ± 8.0	61.1 ± 5.9
	IVC size supine	1.7 ± 0.3	2.3 ± 0.4

Anthropometrics, spirometry, and comprehensive cardiac ultrasound data are presented as averages ± SDs for the participants that completed the shallow and deep dive profiles. n females, males indicates the number of females and males that completed each dive profile,

and n PFO+, PFO- indicates the number of PFO+ and PFO- participants that completed each dive profile. FVC = forced vital capacity; FEV_1 = forced expiratory volume in one second; FEV_1/FVC = the ratio of FVC to FEV_1; FEF_{25-75} = mid-expiratory flow; RV wall = right ventricular wall thickness; IVSd = interventricular septum thickness at end diastole; LVIDd = left ventricular internal dimension at end diastole; LVIDs = left ventricular internal dimension at end systole; LVPWd = left ventricular posterior wall thickness at end diastole; LA s = left atrial size; PV pk vel = pulmonic valve peak velocity; LVOT vel = left ventricular outflow tract velocity; AoV vel = aortic valve velocity; AoV VTI = aortic valve velocity time integral; AVA = aortic valve area; MV-E vel = mitral valve ejection velocity; MV-A vel = mitral valve acceleration velocity; LAT e' = early diastole velocity waveform; E/e' ratio = ratio of E wave to e' wave; RVED area = right ventricular end diastolic area; RVES area = right ventricular end systolic area; %RVEF = right ventricular ejection fraction; LVED vol = left ventricular end diastolic volume; LVES vol = left ventricular end systolic volume; HR at LV vol meas = heart rate at the left ventricular volume measurement; %LVEF = left ventricular ejection fraction; IVC size supine = diameter of the inferior vena cava.

An initial comprehensive echocardiography (Vivid iq, GE HealthCare, Chicago, IL) screening and bubble study was performed on all participants as previously published (Lovering & Goodman, 2012; Elliott *et al.*, 2013). Briefly, an IV catheter was placed in a peripheral vein for injection of saline contrast with and without a Valsalva maneuver to confirm the presence or absence of a PFO. The release of a Valsalva maneuver is not required to open a PFO but instead creates conditions where right atrial pressure exceeds left atrial pressure transiently as bubbles

appear in the right side of the heart while we are simultaneously imaging so that we can see if bubbles travel to the left side of the heart during these optimal conditions. We considered a PFO to be present if bubbles appeared in the left ventricle ≤3 cardiac cycles following right heart opacification with saline contrast. Participants with no bubbles detected in the left ventricle were considered to have no PFO and were assigned to the PFO- group. Participants with bubbles in the left ventricle within 3 cardiac cycles, regardless of how many bubbles, were considered to have a PFO and assigned to the PFO+ group. Of the 50 participants included in the present study, 20 had a PFO and 26 did not have a PFO. Four participants did not have PFO screening performed due to technical difficulty imaging. After PFO screening, a comprehensive echocardiogram was performed on all participants according to standards set by the American Society of Echocardiography to confirm normal cardiac function (Mitchell *et al.*, 2019). Data from the comprehensive echocardiogram are presented in Table 8.

Dive protocol & study design

Participants completed two dives on back-to-back days, with the exception of one participant whose dives were two days apart due to scheduling conflicts. Half of the participants completed their dives in the morning (approximately 10:45 dive start time), and half of the participants completed their dives in the afternoon (approximately 15:30 dive start time). Prior to the dive, participants had resting, baseline measurements made (see "Measurements" below). Then, participants took a boat to the dive site (approximately 15-30 minute boat ride) and completed their dive. Participants were instructed to maintain one of two dive profiles – 18 meters of seawater for 47 minutes, or 30 meters of seawater for 20 minutes. These times were chosen as the longest duration at these depths that were within recreational non-decompression dive limits. The two dive depths were chosen to represent a more moderate dive profile (18

meters) and a more aggressive dive profile (30 meters). The studies reporting SCUBA-induced increases in pulmonary pressure utilized a 30 meter dive profile for 30 minutes (Dujić *et al.*, 2006; Marabotti *et al.*, 2013). 26 participants completed the 18-meter dive protocol, and 24 participants completed the 30-meter dive protocol. 3 participants completed both dive protocols. All participants wore a dive computer (SCUBAPro Mantis M2, El Cajon, CA) so that they could monitor their dive depth and bottom time, and all participants dove with at least one dive buddy. Upon resurfacing from the dive, participants were given sildenafil (50 mg, p.o.) or placebo in a randomized and balanced crossover design for females and males. After waiting 1 hour for the peak effect of the drug, post-dive measurements were made (see '*Measurements'* below). Participants did not take sildenafil prior to the dive to avoid potential risks associated with a systemic vasodilator underwater.

Measurements

All measurements described below were made at four time points – pre-dive 1, post-dive 1, pre-dive 2, and post-dive 2. Measurements included cardiac ultrasound and venous blood draws.

Cardiac ultrasound. Cardiac ultrasound measures included various right heart measures (RVOT VTI, RVOT AT, RVOT ET, RVED area, RVES area, RV dim base and RV dim mid, and TAPSE) as well as various measures used to calculate PASP, Q, and TPR (*v*, IVC collapsibility, IVC size, LVOT diameter, LVOT VTI, and heart rate measured with a 3-lead ECG). All measurements were made in triplicate and triplicate values averaged.

IVC size and collapsibility were used to estimate P_{RA} according to standards set by the American Society of Echocardiography (Lang *et al.*, 2015; Mitchell *et al.*, 2019). IVC collapsibility was calculated as the percentage difference between the maximal and minimal IVC

size upon a rapid sniff. If the IVC diameter was less than 2.1cm and the IVC had greater than 50% collapsibility, P_{RA} was assigned a value of 3mmHg. If the IVC diameter was greater than 2.1cm and the IVC had greater than 50% collapsibility, P_{RA} was assigned a value of 8mmHg. If the IVC diameter was less than 2.1cm and the IVC had less than 50% collapsibility, P_{RA} was assigned a value of 8mmHg. Lastly, if the IVC diameter was greater than 2.1cm and the IVC had less than 50% collapsibility, P_{RA} was assigned a value of 15mmHg (Bamira & Picard, 2018).

To calculate PASP, we applied *v* and the estimated P_{RA} to the modified Bernoulli equation ($PASP = 4v^2 + P_{RA}$) as we and others have previously done (Yock & Popp, 1984; Himelman *et al.*, 1989*b*; Rudski *et al.*, 2010; Duke *et al.*, 2020; DiMarco *et al.*, 2021). Stroke volume was calculated by using LVOT diameter and VTI (Tan *et al.*, 2017). The LVOT diameter was divided by two to find the radius, then squared and multiplied by pi to determine cross-sectional area (CSA) of the LVOT. LVOT CSA was then multiplied by LVOT VTI to obtain stroke volume. Stroke volume was multiplied by heart rate to determine Q. TPR was calculated as the PASP divided by Q, as before (Foster *et al.*, 2014; DiMarco *et al.*, 2021).

Due to technical difficulty imaging, a small number of data points were unobtainable. For those assigned to the shallow dive profile, two participants were missing LVOT VTI measurements post-dive 1 and therefore do not have Q or TPR data at that time point. P_{RA} was unable to be measured in one participant assigned to the deep dive profile, so PASP and therefore TPR were unable to be calculated at all four time points. An additional participant assigned to the deep dive profile could not have *v* measured post-dive 2 and is therefore missing PASP and TPR measurements at that time point. One last participant assigned to the deep dive profile could not have *v* measured at any time point and therefore does not have any PASP or TPR data.

While the radius of the vessel is the most important factor in regulating TPR, blood viscosity is an additional variable that should be taken into consideration (Hoffman, 2011; Vanderpool & Naeije, 2018). To correct for blood viscosity, TPR was corrected for hematocrit (HCT). A reference HCT (HCTr) of 0.45 was used to normalize all measured TPR irrespective of their measured HCT (HCTm). The measured TPR was divided by $e^{2(HCTr - HCTm)}$ (Vanderpool & Naeije, 2018). The HCT corrected TPR was multiplied by Q to obtain HCT corrected PASP. The HCT corrected values of PASP and TPR are reported and used for this study.

Venous blood draws and processing. An IV catheter was placed into an antecubital vein for obtaining blood draws. At all four time points, a 15mL venous blood sample was drawn from the IV catheter into serum separator tubes (SSTs; gold or tiger top tubes pre-filled with a polyester-based gel with silica particles that act as a clot activator; Becton-Dickinson, Franklin Lakes, NJ). A small venous blood sample was also drawn into a pre-heparinized 3mL syringe at all time points, and a microcapillary tube was filled to measure HCT.

Due to technical difficulties with the IVs, a small number of blood samples were unobtainable. There are no missing blood samples from those that completed the shallow dive profile. For those that completed the deep dive profile, there was one blood sample missing from the post-dive 1 time point, one blood sample missing from the pre-dive 2 time point, and one blood sample missing from the post-dive 2 time point.

All venous blood samples drawn into SSTs were allowed to sit at room temperature for at least 30 minutes to fully clot, after which they were centrifuged at 1500g for 10 min. Serum was separated and frozen at -20°C for up to 2 weeks while at the dive site in Bol, Island of Brac, Croatia and then transferred from -20°C to a -80°C freezer until analyzed. Venous blood drawn for HCT was analyzed immediately by centrifuging at 10,000 rpm for 10 minutes.

Cytokine analysis

Frozen serum samples were thawed fully while refrigerated on ice. Samples were analyzed using a multi-analyte flow assay kit as described in the manual (BioLegend LEGENDplex Human Inflammation panel 1). Due to the number of samples, multiple assays were used for analysis, but all samples from a given participant were analyzed within the same assay. Briefly, samples were incubated with 13 different capture beads: IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Samples were diluted twofold prior to analysis. All samples were analyzed via flow cytometry (BD Biosciences Accuri C6) according to manufacturer guidelines (Biolegend.com/legendplex). All samples were run in duplicate and duplicates averaged. Upon analysis, any duplicate sample with a coefficient of variation (CV) > 30% was excluded from analysis, per suggestion in the manufacturer manual. Table 3 documents the number of samples excluded for having high CVs. Samples below limits of detection were not excluded from analysis, but the number of samples below limits of detection are documented in Table 9. Concentrations below limits of detection were calculated from the extrapolated standard curve. Lower limits of detection were calculated as three times the standard deviation of the mean fluorescence intensity of the blank.

Statistical analysis

Statistical analyses were done using GraphPad Prism v.9.4.1 with alpha set *a priori* to be equal to 0.05. PASP, Q, TPR, and right heart measures were analyzed via 2-way repeated measures mixed effects analyses with time (pre- to post-dive) and drug (sildenafil and placebo) as the two factors. The absolute change in PASP, Q, and TPR were also analyzed with a 2-way repeated measures mixed effects analysis with drug (sildenafil and placebo) and PFO (PFO- and

PFO+) as the two factors. The two dive profiles were analyzed independently. All post-hoc testing was done with the Sidak post-hoc test.

Inflammatory cytokine concentrations were all initially analyzed via 2-way repeated measures mixed effects analyses between time (pre- to post-dive) and drug (sildenafil and placebo) with Sidak post-hoc analysis within each dive profile. Upon confirmation that no cytokine concentrations were affected by sildenafil, inflammatory cytokine concentrations were analyzed via paired t-tests pre- to post-dive within each day.

RESULTS

Participant characterization

Spirometry and comprehensive echocardiography are presented in Table 8 to confirm normal cardiopulmonary and respiratory function in participants. In addition, right heart measures were taken at all four time points to determine if changes in pulmonary pressure had any measurable effects on right heart function (Table 10). All values were within normal ranges (Lang *et al.*, 2015; Mitchell *et al.*, 2019), and the right heart measures did not change pre to post-dive when participants received placebo and were not altered by sildenafil (Table 10).

Effect of SCUBA diving, sildenafil, and PFO on pulmonary pressure

PASP did not increase in response to SCUBA diving when participants received a placebo (Fig 16). This pattern was consistent regardless of the dive profile, as neither dive profile evoked an increase in PASP when participants took a placebo (Fig 16). Similarly, Q and TPR did not increase in either dive profile when participants took the placebo (Fig 16). However, there was a main effect of the drug treatment on TPR during the shallow dive profile, but there were no significant pairwise differences (Fig 16E). Additionally, there was an interaction between the

drug treatment and time when participants completed the deep dive profile, but there were no significant pairwise differences (Fig 16F).

There was no effect of a PFO on PASP or Q during either dive profile (Fig 17). During the deep dive profile, there was similarly no effect of a PFO on TPR (Fig 17F). However, there was an interaction between the presence of a PFO and drug treatment on TPR when participants completed the shallow dive profile (Fig 17E). When participants received sildenafil, PFO+ participants had a greater increase in TPR compared to PFO- participants (Fig 17E).

Inflammatory response to SCUBA diving

The use of sildenafil did not affect the concentrations of any cytokines during the shallow or deep dive profile (data not shown), so cytokine data are presented pre and post-dive within dive 1 and within dive 2. When divers completed the shallow dive profile, IL-6 concentration increased post-dive 1 ($p = 0.0057$; Fig 18A). Both IL-6 ($p = 0.0045$) and MCP-1 ($p = 0.0005$) increased post-dive 2 (Fig 18C & 18D) for participants that completed the shallow dive profile. No cytokines increased post-dive during the deep dive profile on day 1 (Fig 19A & 19B). However, IL-6 ($p = 0.0460$) and MCP-1 ($p = 0.0434$) increased while IL-23 ($p = 0.0145$) decreased post-dive for those that completed the deep profile (Fig 19C & 19D).

Table 9. Inflammatory cytokine data from Aim 3.

		Pre dive 1			Post dive 1			Pre dive 2			Post dive 2		
	LLOD range	n < LLOD	n CV>30%	n included	n < LLOD	n CV>30%	n included	n < LLOD	n CV>30%	n included	n < LLOD	n CV>30%	n included
IL-1β (pg/mL)	0.78 - 4.55	5	0	50	9	0	49	9	0	49	8	0	49
IFN-α2 (pg/mL)	0.32 - 4.33	0	0	50	0	0	49	0	0	49	0	0	49
IFN-γ (pg/mL)	0.22 - 5.03	7	0	50	7	0	49	8	0	49	8	0	49
TNF-α (pg/mL)	0.42 - 4.37	5	0	50	3	0	49	4	0	49	3	0	49
MCP-1 (pg/mL)	0.24 - 6.28	0	0	50	0	0	49	0	0	49	0	0	49
IL-6 (pg/mL)	0.22 - 2.47	1	0	50	0	0	49	1	0	49	0	0	49
IL-8 (pg/mL)	0.29 - 29.36	8	0	50	5	1	48	6	1	48	6	0	49
IL-10 (pg/mL)	0.27 - 5.61	1	0	50	2	0	49	2	0	49	2	0	49
IL-12p70 (pg/mL)	0.34 - 2.91	3	0	50	4	0	49	5	0	49	4	0	49
IL-17A (pg/mL)	0.06 - 0.78	1	0	50	4	0	49	2	0	49	2	0	49
IL-18 (pg/mL)	0.37 - 12.03	0	0	50	0	0	49	0	0	49	0	0	49
IL-23 (pg/mL)	0.29 - 14.08	4	0	50	5	0	49	4	0	49	6	0	49
IL-33 (pg/mL)	2.44 - 104.87	3	0	50	3	0	49	4	0	49	5	0	49

The range of lower limits of detection (LLOD range) for the assays used in the study are provided. The number of samples below lower limits of detection (n < LLOD) as well as samples excluded for high coefficients of variation (n CV > 30%) are provided from each of the four time points inflammatory cytokine concentrations were measured. The total number of samples analyzed per time point from both the shallow and deep profiles is included (n included).

Table 10. Right heart function pre and post SCUBA diving.

	Shallow profile				Deep profile			
	Sildenafil		Placebo		Sildenafil		Placebo	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
RVOT VTI	18.47 ± 2.95	17.57 ± 2.41	18.61 ± 2.72	18.07 ± 3.26	18.26 ± 3.73	18.77 ± 3.25	18.57 ± 2.41	18.01 ± 2.37
RVOT AT (ms)	140.96 ± 27.87	140.38 ± 31.52	146.88 ± 27.15	141.00 ± 21.09	139.91 ± 21.68	132.36 ± 19.83	139.96 ± 25.62	136.65 ± 24.02
RVOT ET (ms)	312.96 ± 30.65	293.88 ± 25.81	311.65 ± 40.78	297.68 ± 25.62	304.86 ± 39.94	295.59 ± 40.58	308.91 ± 33.66	313.22 ± 34.73
RVED area	18.94 ± 3.82	18.23 ± 4.15	19.11 ± 4.20	18.75 ± 3.42	25.36 ± 3.94	24.96 ± 4.30	26.07 ± 3.63	25.67 ± 4.11
RVES area	12.73 ± 3.13	11.32 ± 2.66	12.03 ± 2.11	11.91 ± 2.42	13.89 ± 2.46	13.20 ± 2.64	13.83 ± 2.64	14.00 ± 2.62
RV dim base (cm)	3.71 ± 0.51	3.51 ± 0.42	3.65 ± 0.38	3.62 ± 0.40	4.23 ± 0.48	4.17 ± 0.49	4.32 ± 0.47	4.33 ± 0.49
RV dim mid (cm)	2.67 ± 0.48	2.68 ± 0.45	2.63 ± 0.47	2.67 ± 0.42	3.36 ± 0.29	3.19 ± 0.30	3.34 ± 0.33	3.36 ± 0.30
TAPSE (cm)	2.45 ± 0.45	2.36 ± 0.32	2.47 ± 0.33	2.42 ± 0.40	2.45 ± 0.38	2.35 ± 0.38	2.46 ± 0.27	2.33 ± 0.32

Additional measurements of right heart function were made pre and post SCUBA diving on both dive days. Data are presented for both shallow and deep dive profile groups. RVOT VTI = right ventricular outflow tract velocity time integral; RVOT AT = right ventricular outflow tract acceleration time; RVOT ET = right ventricular outflow tract ejection time; RVED area = right ventricular end diastolic area; RVES area = right ventricular end systolic area; RV dim base = right ventricular basal diameter; RV dim mid = right ventricular mid diameter; TAPSE = tricuspid annular plane systolic excursion.

DISCUSSION

Our results suggest that non-decompression SCUBA diving does not result in an increase in PASP or TPR but can increase concentrations of some inflammatory cytokines. Sildenafil did not reduce PASP or TPR, but we most likely observed a lack of effect of sildenafil because SCUBA diving did not evoke increases in PASP and/or TPR. Accordingly, the exact role of NO in regulating pulmonary vascular tone post SCUBA diving remains unknown. Interestingly, the presence of a PFO may alter the effects of sildenafil on TPR, although this observation was not consistent between dive profiles and more work should be done to determine whether this is a repeatable finding.

Lastly, because PASP and/or TPR did not increase with either dive profile in the whole group or in just PFO+ or PFO- participants, we did not examine relationships between inflammation and pulmonary pressure. Therefore, the role of cytokines in modulating pulmonary pressure remains unknown, and future work should examine this relationship with SCUBA dive profiles that cause increased PASP and TPR.

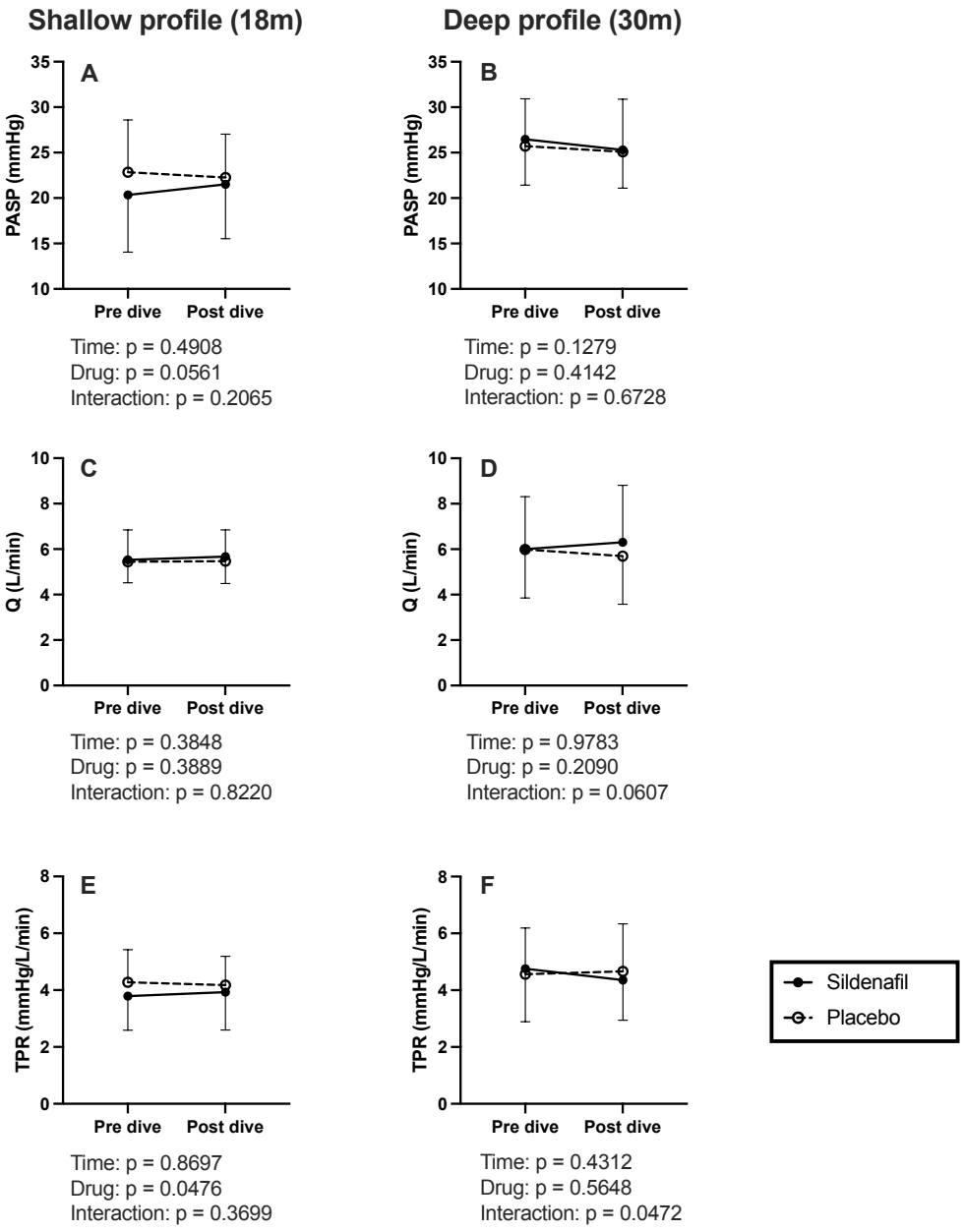


Figure 16. PASP, Q, and TPR pre and post SCUBA diving with sildenafil and placebo. PASP (mmHg), Q (L/min), and TPR (mmHg/L/min) are shown pre- and post-dive when participants received sildenafil and placebo for both dive profiles. Sildenafil is represented with closed circles and solid lines and placebo with open circles and dashed lines. Main effects of time (pre- to post-dive) and drug (sildenafil and placebo) as well as interactions

between the two are provided below each panel. A) PASP over time and between drug treatments for the shallow dive profile; B) PASP over time and between drug treatments for the deep dive profile; C) Q over time and between drug treatments for the shallow dive profile; D) Q over time and between drug treatments for the deep dive profile; E) TPR over time and between drug treatments for the shallow dive profile; F) TPR over time and between drug treatments for the deep dive profile.

The effect of decompression on pulmonary vascular tone regulation

The previous studies that have shown an increase in pulmonary pressure post SCUBA diving utilized a decompression dive profile of 30 meters for 30 minutes (Dujić *et al.*, 2006; Marabotti *et al.*, 2013), so the negative PASP and TPR results of the present study may be at least partially attributed to the use of non-decompression dive profiles. Even more interesting is that pulmonary pressure does not appear to simply be a factor of dive depth up to 30 meters of sea water for 20 minutes. This further supports the idea that there is something fundamental to decompression dives that causes an increased pulmonary pressure.

During SCUBA diving, inert gases become dissolved in tissues relative to the degree of increased ambient pressure. Decompression refers to the reduction in ambient pressure a diver experiences during an ascent that allows those dissolved gases to escape solution, which have the potential to form venous gas emboli. The professional association of diving instructors (PADI) created guidelines for safe diving practices with the intention of minimizing the formation of venous gas emboli during decompression, which pose the potential to cause decompression sickness. However, many studies find venous gas emboli even when safe diving practices are

utilized, and the venous gas emboli are more common in the more aggressive decompression dive profiles (Marabotti *et al.*, 1999, 2013; Dujic *et al.*, 2005*a*, 2005*b*; Dujić *et al.*, 2006).

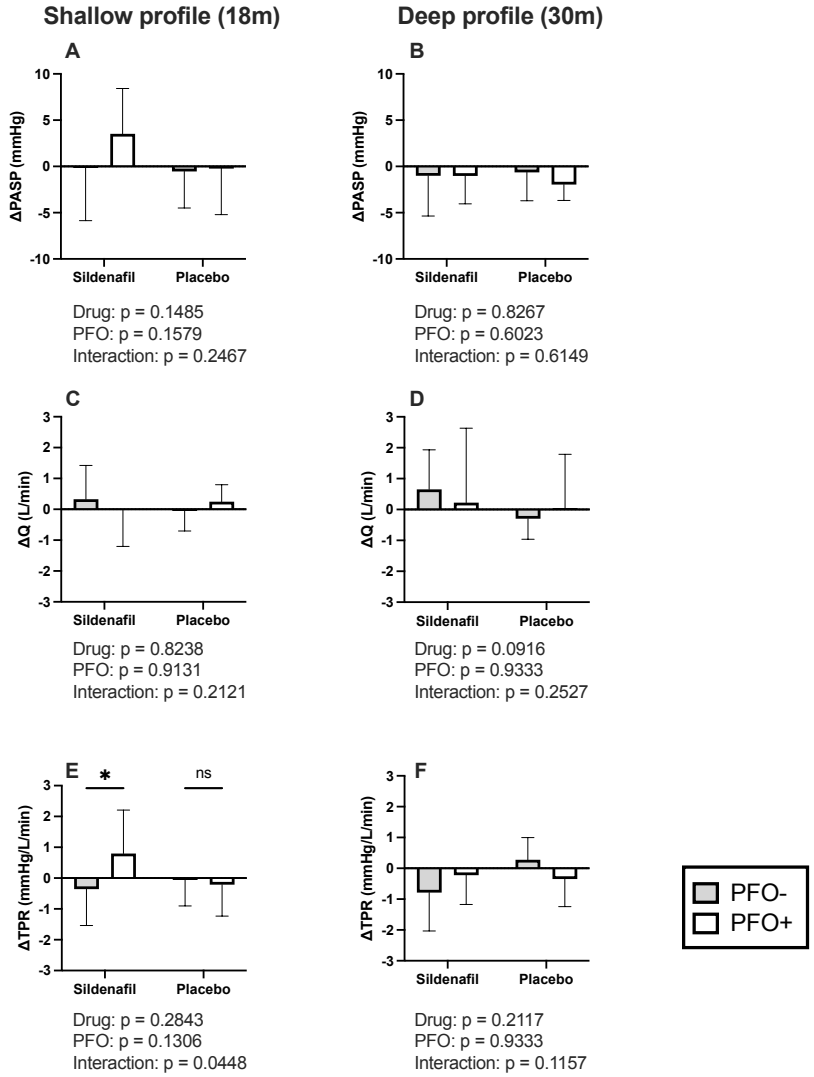


Figure 17. Changes in PASP, Q, and TPR between drug and PFO. The absolute change in PASP, Q, and TPR are shown between PFO- (gray bars) and PFO+ (white bars) participants when they received sildenafil and placebo. Main effects of the drug (sildenafil and placebo) and PFO (PFO- and PFO+) as well as interactions between the two are provided below each panel. A) The change in (Δ) PASP between drug treatments and PFO

for the shallow dive profile; B) The change in (Δ) PASP between drug treatments and PFO for the deep dive profile; C) The change in (Δ) Q between drug treatments and PFO for the shallow dive profile; D) The change in (Δ) Q between drug treatments and PFO for the deep dive profile; E) The change in (Δ) TPR between drug treatments and PFO for the shallow dive profile; F) The change in (Δ) TPR between drug treatments and PFO for the deep dive profile. Bars indicate pairwise comparisons. * indicates p < 0.05.

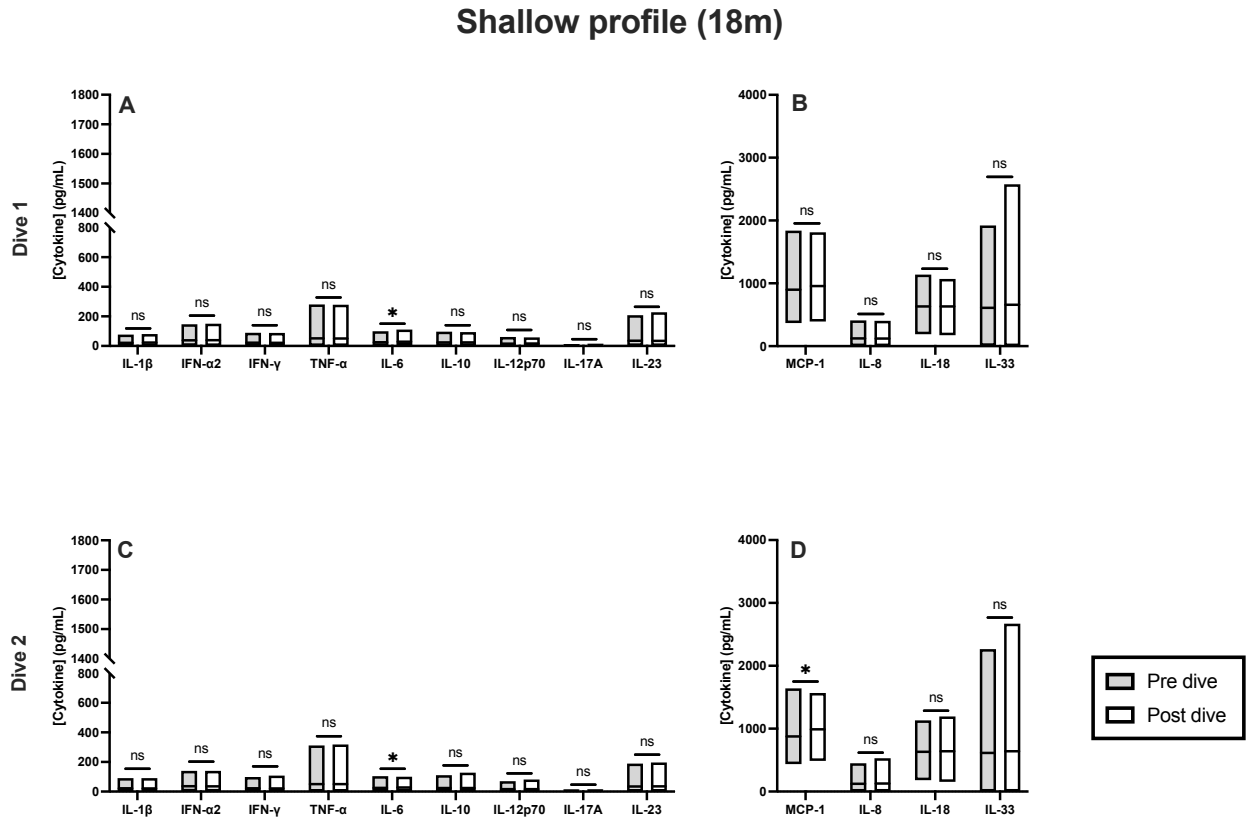


Figure 18. Inflammation in response to shallow dive profile. Concentrations of 13 inflammatory cytokines pre and post SCUBA dive on days 1 and 2 are shown for participants that completed the shallow dive profile. Panels A and B show cytokine concentrations pre and post-dive 1, and panels C and D show cytokine concentrations pre and post-dive 2. Bars represent the range of data, and the line within each bar represents

the group means. Pre dive values are in gray bars, and post-dive values are in white bars. * indicates p < 0.05 comparing post-dive to pre-dive values.

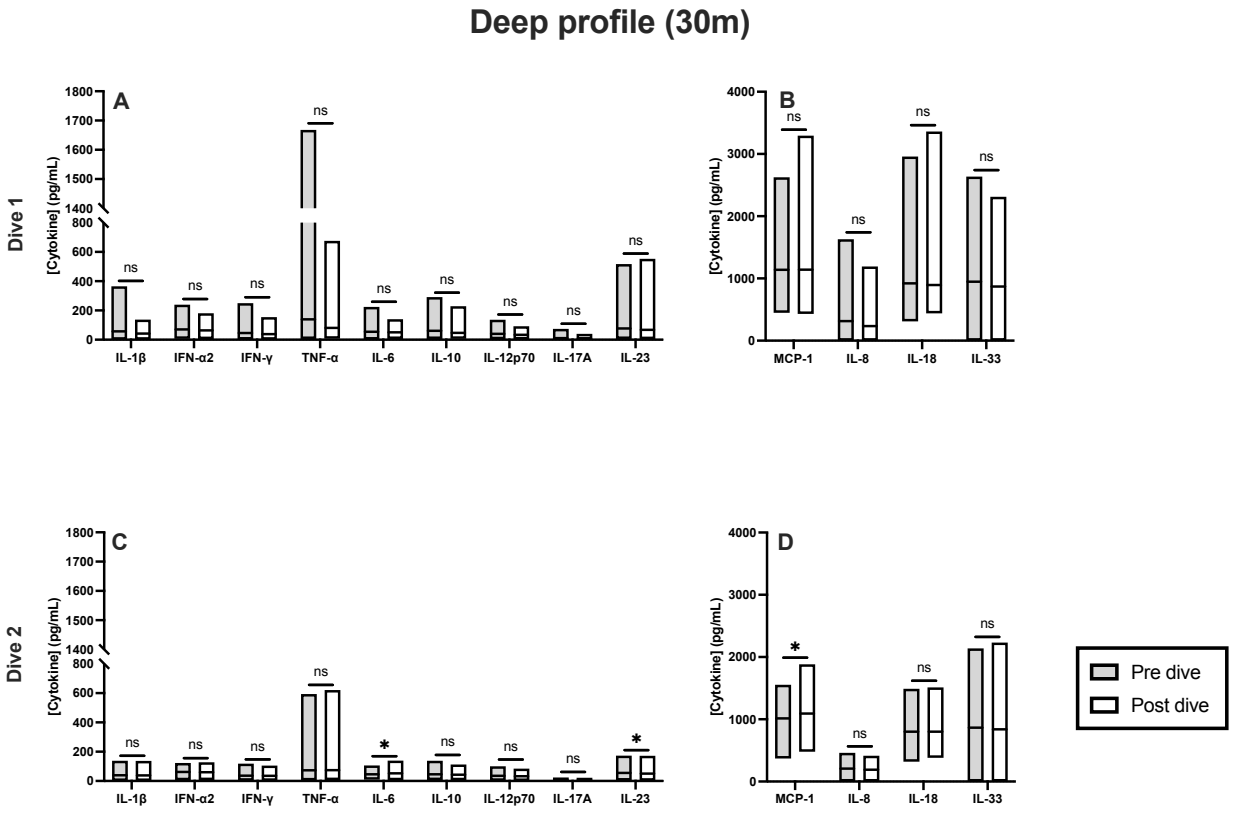


Figure 19. Inflammation in response to deep dive profile. Concentrations of 13 inflammatory cytokines pre and post SCUBA dive on days 1 and 2 are shown for participants that completed the deep dive profile. Panels A and B show cytokine concentrations pre and post-dive 1, and panels C and D show cytokine concentrations pre and post-dive 2. Bars represent the range of data (absolute maximum and minimum values) and the line within each bar represents the group means. Pre dive values are in gray bars, and post-dive values are in white bars. * indicates p < 0.05 comparing post-dive to pre-dive values.

It is possible that venous gas emboli are at least partially responsible for increasing pulmonary pressure, which could explain differences in the pulmonary pressure response between decompression and non-decompression dives, although the literature on this is mixed. In support of venous gas emboli causing increased pulmonary pressure, Marabotti *et al.* (2013) found that PASP increased only in those participants that produced venous gas emboli upon resurfacing. Additionally, in a pig model, the greatest increases in pulmonary pressure were associated with the greatest production of venous gas emboli (Vik *et al.*, 1993). Conversely, Valic *et al.* (2005) found that venous gas emboli are not responsible for increasing pulmonary pressure. Valic *et al.* (2005) used a hyperbaric chamber meant to simulate an 18-meter dive at varying times that represented both decompression and non-decompression dive times. They found that pulmonary pressure did not increase at any time spent at the simulated 18 meters despite the presence of significant venous gas emboli (Valic *et al.*, 2005).

Importantly, hyperbaric chambers do not fully capture the conditions experienced during a SCUBA dive, which also include immersion, cold, and mild exercise. Therefore, hyperbaria alone and venous gas emboli produced during decompression may not be directly responsible for increasing pulmonary pressure. Rather, water immersion, cold, or a combination of these factors experienced during a SCUBA dive that get more extreme during decompression dives may be responsible for increasing pulmonary pressure. This could explain the association between venous gas emboli and pulmonary pressure observed by Marabotti *et al.* (2013) during a real world SCUBA dive and the lack of association between venous gas emboli and pulmonary pressure observed by Valic *et al.* (2005) in a simulated (dry) SCUBA dive. In support of this, water immersion independent of SCUBA diving, particularly in cold water, can cause immersion-induced pulmonary edema, which is characterized by elevated mean pulmonary

artery pressure and pulmonary wedge pressure (Moon *et al.*, 2016; Martina *et al.*, 2017). Therefore, cold water immersion rather than the venous gas emboli produced during decompression may be more significant in regulating pulmonary pressure. However, this does not explain why pulmonary pressure remains elevated hours after a dive occurs when immersion is no longer a factor, but some speculations on the links between venous gas emboli, endothelial dysfunction, immune system activity, and pulmonary pressure regulation are discussed below.

Effect of SCUBA diving on inflammation

The present study found that MCP-1 and IL-6 concentrations increased post-dive in both non-decompression dive profiles utilized. These results are supported by other studies showing increased immune system activity post SCUBA diving, including increased circulating granulocytes (Marabotti *et al.*, 1999), increased neutrophil counts and activation (Nossum *et al.*, 2002; Thom *et al.*, 2013; Madden *et al.*, 2014), increased inflammatory cytokine expression (Žarak *et al.*, 2021; Rocco *et al.*, 2021), and upregulation of genes and mRNA related to the immune response (Eftedal *et al.*, 2013; Sureda *et al.*, 2014). Rocco *et al.* (2021) specifically found that IL-6 concentrations increased and MCP-1 concentrations trended towards increasing in their decompression dive profile of 50 meters of seawater for 20 minutes, and Žarak *et al.* (2021) similarly found increased IL-6 after SCUBA diving to 20-30 meters of seawater for 30 minutes.

It is not unexpected that the only cytokines found to increase were MCP-1 and IL-6, as these cytokines are typically involved in immediate acute responses to other stressors. MCP-1 increases within minutes of hypoxic exposure in rats (Chao *et al.*, 2011) and humans (Mishra *et al.*, 2016). Similarly, IL-6 is typically the only cytokine that increases immediately post exercise (Pedersen *et al.*, 2001; Petersen & Pedersen, 2005*b*; Banzet *et al.*, 2012) in order to stimulate an

increase or decrease in additional cytokines. Had we measured cytokine concentrations later than approximately 1-2 hours post-dive, it is possible we would have observed greater increases in other cytokines downstream of IL-6 and MCP-1.

Endothelial dysfunction, immune activation, and pulmonary pressure regulation

In addition to immune activation, SCUBA diving and hyperbaric SCUBA simulations can induce endothelial dysfunction in both human and animal models (Nossum *et al.*, 1999, 2002; Brubakk *et al.*, 2005; Madden *et al.*, 2010; Culic *et al.*, 2014). There is support that venous gas emboli are responsible for the endothelial dysfunction (Nossum *et al.*, 1999, 2002) and that in turn the endothelial dysfunction is responsible for the increased immune activity observed post SCUBA diving. One study showed that the endothelial dysfunction secondary to gas bubbles resulted in no visible endothelial damage but did induce increases in neutrophil count (Nossum *et al.*, 2002). Additionally, Marabotti *et al.* (1999) found that in those that produced venous gas emboli, granulocyte count increased, further supporting the link between SCUBA induced production of venous gas emboli, endothelial dysfunction, and immune activation. It is interesting that both non-decompression dives and decompression dives alike induce these immune changes. This further supports the idea that venous gas emboli, which can be produced in both decompression and non-decompression dives, may be partially responsible for the immune changes due to the induction of endothelial dysfunction.

However, an interesting question is whether the increased immune activity from venous gas emboli-induced endothelial dysfunction is responsible for changes in pulmonary pressure. If this link does exist, we theoretically should have seen an increased pulmonary pressure in the present study that coincided with the increased IL-6 and MCP-1. However, the literature on the role of inflammatory cytokines in modulating acute changes in pulmonary vascular tone are

limited to only a few cytokines, primarily IL-1β and TNF-α (Tsai *et al.*, 2004). Therefore, it is possible that IL-6 and MCP-1 do not modulate acute pulmonary vascular tone. This is supported by Savale *et al.* (2009) who found that IL-6 knockout mice had the same increase in pulmonary pressure in response to hypoxia as wild type mice, indicating that IL-6 does not acutely regulate pulmonary vascular tone. Even if MCP-1 and IL-6 were produced because of endothelial damage induced by venous gas emboli, a more aggressive dive profile may be required to induce changes in more acute acting vasoactive inflammatory cytokines. This could also explain why Valic *et al.* (2005) found venous gas emboli without increased pulmonary pressure – it is possible that the venous gas emboli induced only mild changes in the pulmonary vascular endothelium, which did not produce vasoactive molecules. Therefore, in more aggressive dive profiles, greater changes in vasoactive substances produced secondary to venous gas emboli may be responsible for increasing pulmonary vasoconstriction and pressure, but this remains speculative. More research should be done to directly examine the link between venous gas emboli, pulmonary endothelial dysfunction, production of inflammatory cytokines, and increases in pulmonary pressure.

The role of NO in pulmonary pressure regulation

The present study found that the use of sildenafil did not alter PASP or Q. Additionally, TPR did not decrease when participants received sildenafil in either dive profile, which is contrary to our hypothesis. It is possible that the dose of sildenafil was not strong enough to induce changes in pulmonary resistance. However, 50 mg of sildenafil was sufficient to reduce mean pulmonary artery pressure and pulmonary wedge pressure in those susceptible to immersion pulmonary edema (Moon *et al.*, 2016; Martina *et al.*, 2017). Similarly, this dose was sufficient to reduce the degree of hypoxic pulmonary vasoconstriction (Carter *et al.*, 2019; Kelly

et al., 2022). Therefore, the lack of reduction of TPR is unlikely to be related to the dose of sildenafil chosen and more likely related to the availability of NO.

Interestingly, SCUBA diving does not reduce inducible NO synthase (Sureda *et al.*, 2014) but does reduce endothelin-1 (ET-1), the antagonist of NO (Žarak *et al.*, 2021). Inducible NO synthase also increased 3 hours post-dive from baseline values (Sureda *et al.*, 2014). Therefore, NO availability may not be a limiting factor during or post SCUBA diving, and a lack of NO availability and/or an increase in ET-1 may not be responsible for increasing pulmonary pressure post decompression SCUBA diving. From the current literature and results of the present study, it seems more likely that increased immune activity secondary to endothelial dysfunction is responsible for increased pulmonary pressure rather than a lack of NO availability. However, this is still very speculative, as no studies have definitively shown simultaneous increases in pulmonary pressure and immune activity in humans undergoing a real-world SCUBA dive.

The effect of a PFO

The present study found no effect of a PFO on PASP in either dive profile. This confirms the results of others that show no PFO differences in acute PASP changes with hypoxia (Duke *et al.*, 2020; DiMarco *et al.*, 2021). However, an interesting observation is that the presence of a PFO altered the effect of sildenafil treatment on TPR. Contrary to our hypothesis, PFO+ participants increased TPR when they received sildenafil, and the increase in TPR was significantly greater than PFO- participants receiving sildenafil. Any potential mechanisms that could cause sildenafil to increase TPR only in those with a PFO are entirely unknown. Additionally, this was only observed during the shallow dive profile, so more work needs to be done to determine if this is a repeatable finding or simply a result of the participants studied.

Limitations

A primary limitation to the present study, as well as all studies on SCUBA diving physiology, is that measurements cannot be made during the SCUBA dive itself. It would be extremely interesting to directly measure pulmonary blood volume and pressure during a SCUBA dive to determine whether or not the changes in cardiopulmonary function only occur post SCUBA diving or whether those changes begin during the dive. Lastly, the measures of PASP and Q obtained in the present study are indirect measures, the limitations of which are discussed in greater detail by (Parasuraman *et al.*, 2016). Nevertheless, using this approach, we have demonstrated that we can detect differences between groups (Norris *et al.*, 2014*b*; Laurie *et al.*, 2018) when those differences have been shown to exist using direct catheterization measures (Kovacs *et al.*, 2012; Goss *et al.*, 2018).

Conclusions

Overall, we found that two different non-decompression dive profiles resulted in no increases in PASP or TPR. Sildenafil did not reduce PASP or TPR in either dive profile, but because our dive profiles did not increase PASP, the role of NO in regulating pulmonary vascular tone is inconclusive. Studies employing a dive protocol that induces greater increases in PASP and/or TPR should examine whether NO is a potential vasoactive substance responsible for the changes in pulmonary pressure observed following SCUBA diving requiring decompression. Lastly, future work should examine intraindividual responses to various decompression and non-decompression dive protocols to determine whether there are inherent differences between decompression and non-decompression SCUBA diving on pulmonary pressure and immune responses to SCUBA diving and whether there is a link between venous gas emboli, endothelial dysfunction, immune activity, and pulmonary pressure regulation.

CHAPTER VII

CONCLUSIONS

MAIN FINDINGS

Most studies examining cardiopulmonary regulation opt to focus solely on the cardiovascular and respiratory systems and largely ignore potential systemic influences from other physiologic systems or modulatory factors such as the immune system, iron availability, and cardiopulmonary anomalies. The three studies in this dissertation all had primary cardiopulmonary outcome measures alongside secondary outcome measures that examined the role of iron status parameters, immune system components, and the presence or absence of a PFO as modulators of the primary cardiopulmonary responses and variables. The findings from this dissertation on the roles of these modulatory variables are mixed, with some cardiopulmonary variables exhibiting a degree of association with these variables while others did not.

Specifically, in Chapter IV, we show that COi and HWI can stimulate a similar increase in EPO independently and when combined. An unexpected but significant finding from this study was that females had greater increases in EPO in response to COi, HWI, and combined COi and HWI, and this can only be partially explained by sex differences in the stimulus for EPO production. However, contrary to our hypothesis, inflammatory cytokine concentrations and iron availability in these otherwise healthy humans were not associated with the large degree of variability in EPO concentrations in response to the CO and heat interventions.

In chapter V, we examined the relationships between iron status parameters, various immune system components, and the presence or absence of a PFO with sea level Hb mass. Ferritin and transferrin, which indicate iron storage and transport, respectively, were the

strongest predictors of Hb mass. Other studies have shown similar significant relationships between ferritin and Hb mass (Garvican *et al.*, 2012; Govus *et al.*, 2015; Schmidt *et al.*, 2020; Goodrich *et al.*, 2020; Koivisto-Mørk *et al.*, 2021), but we are the first to show that transferrin may be just as important of a predictor of Hb mass. While we do show some significant linear relationships between immune system components, primarily IFN-α2 and MCP-1 concentrations and neutrophil cell counts, with Hb mass, these relationships all had R^2 values below 0.10 and therefore do not predict a large degree of the variability in Hb mass. Contrary to our hypothesis, we show that the presence of a PFO did not alter sea level Hb mass, but PFO+ men did exhibit lower ferritin concentration. Additionally, the presence of a PFO altered the slope of the linear relationship between some iron and immune variables with Hb mass.

Rather than examining red blood cell regulation, Chapter VI examined pulmonary vascular tone regulation in response to recreational SCUBA diving and determined whether NO availability, inflammatory cytokine concentrations, and the presence or absence of a PFO were potential modulators of this response. However, contrary to our hypothesis, two different non-decompression SCUBA diving profiles did not elucidate increases in pulmonary pressure or resistance on average. Despite this observation, we still expected sildenafil, a drug that increases NO availability, to reduce pulmonary pressure and resistance from pre-dive values, a finding that we did not observe, indicating that NO availability may not regulate pulmonary vascular tone during SCUBA diving. However, if SCUBA diving did not reduce NO bioavailability, it is possible that this is why sildenafil did not alter PASP or TPR. Additionally, PFO+ participants did not have greater increases in PASP or TPR, data which supports our hypothesis. These findings add to the growing body of work reporting that the presence of a PFO is not associated with altered acute pulmonary pressure regulation in otherwise healthy humans with

environmental challenges such as hypoxia (Duke *et al.*, 2020; DiMarco *et al.*, 2021). Lastly, because PASP, TPR, and most cytokines did not increase with SCUBA diving, the exact role of inflammatory cytokines in modulating pulmonary vascular tone regulation post SCUBA diving is still unknown.

SUMMARY AND FUTURE DIRECTIONS

One of the most intriguing findings from Chapter IV were the sex differences in EPO responsiveness. There were sex differences in the CO stimulus, with females having greater peak HbCO than males, which can partially explain these unexpected results. However, there were no sex differences in any of the stimuli involved in HWI. Future work should examine potential mechanisms underlying these sex differences in EPO, particularly in response to acute heat stress. More direct measurements of the stimuli, such as taking arterial blood samples to directly measure CaO_2 and measuring Q_R instead of renal blood velocity, would be useful in this quantification. In addition, while we found sex differences in EPO in response to acute heat stress, Lundby *et al.* (2023) do not suggest that there are sex differences in increases in Hb mass in response to heat acclimation. In those studies that do show sex differences in chronic increases in Hb mass, males typically have greater increases in Hb mass than females (Nummela *et al.*, 2021). Therefore, the work provided in this dissertation combined with the literature on chronic Hb mass regulation leaves a large gap in the literature. Namely, there may be sex differences in the translation from EPO to viable red blood cells, and work should be done to explore whether this is true and what that mechanism may be.

One of the primary limitations in Chapter V of this dissertation is that it was not an interventional study, but this limitation also allows for many follow-up studies. We provided novel information characterizing the relationships between iron status parameters, various

immune system components, the presence or absence of a PFO, and Hb mass, and this data provides useful direction in designing potential interventional studies that could closely examine red blood cell regulation in humans. Directly inducing a systemic low-grade inflammation and/or using a general anti-inflammatory treatment such as an NSAID and monitoring Hb mass changes over weeks would provide useful insight into a more direct regulatory role of the immune system on Hb mass in healthy humans. Additionally, an interesting future study would be to compare Hb mass in those with and without iron deficiency anemia. After, an iron supplement could be employed in those with iron deficiency anemia to determine if Hb mass increases over weeks as a result. Lastly, although we did not find the presence of a PFO altered Hb mass, we did find that the presence of a PFO affected ferritin, so future work should examine how iron regulation is altered by a PFO, whether percutaneous PFO closure can increase ferritin, and why lower ferritin is not seen in females with a PFO.

The main cardiopulmonary outcomes of interest in Chapter VI were pulmonary pressure and resistance post-SCUBA diving, and we found that non-decompression SCUBA diving profiles did not induce increases in these variables as expected. Therefore, the conclusions we can draw about the regulatory or modulatory roles of NO, inflammatory cytokines, and the presence or absence of a PFO are not definitive. The most important future study to come from the work provided in Chapter VI would be to examine the role of these modulatory variables during more aggressive SCUBA diving profiles that induce increases in pulmonary pressure and resistance. The literature suggests a stronger role of the immune system in modulating pulmonary vascular tone than NO during SCUBA diving, so rather than use a drug treatment such as sildenafil, an important next step could be to utilize a general anti-inflammatory treatment post-SCUBA diving to determine whether that reduces the SCUBA diving induced

increase in inflammatory cytokines, and whether that reduces pulmonary pressure. However, that study would need to be performed during a SCUBA dive in which pulmonary pressure and perhaps more vasoactive inflammatory cytokines like TNF-α and IL-1β increase.

Overall, the work provided in this dissertation provides novel and prefatory work regarding the modulatory role of iron status parameters, various immune system components, and the presence or absence of a PFO on red blood cell and pulmonary vascular tone regulation in humans. All of the studies provided within this dissertation answer important cardiopulmonary questions, but they also provide associative relationships between these modulatory variables and the primary cardiopulmonary variables in question. As a whole, future work would benefit from direct interventional studies aimed to increase or decrease immune system activity and/or iron availability. In addition, studying Hb mass and pulmonary pressure regulation pre- and post-percutaneous PFO closure would provide useful insight into the direct role of a PFO in modulating these aspects of the cardiopulmonary system.

CHAPTER VIII

APPENDICES

EQUATIONS APPENDIX

Equation 1: calculation of renal oxygen delivery

4. Renal oxygen delivery (mL O_2/min) = Q_R (L/min) • CaO_2 (mL O_2/L)

Equation 2: calculation of CaO_2

1. CaO_2 = [(1.34 ml O_2/ g Hb) • ([Hb]/100 ml blood) • SaO_2] + [(0.003 ml O_2/ 100 ml blood/ mm Hg) • PaO_2)]

Equation 3: Modified Bernoulli equation

2. PASP = $[4(v)^2]$ + P_{RA} mmHg

Equation 4: calculation of stroke volume

5. Stroke volume = $[(\text{LVOT diameter}/2)^2 \bullet \pi]$ • LVOT VTI

CALCULATIONS APPENDIX

Calculation 1: example calculation of estimated renal oxygen delivery

COi

Baseline

Baseline HbCO = 1.28%

(100 – 1.28)/100 = 0.9872

Renal blood velocity = 44.55 cm/s

Estimated renal oxygen delivery = 43.98 arbitrary units (au)

Post-COi

Peak HbCO = 10.7%

(100 – 10.7)/100 = 0.8930

Minimum renal blood velocity = 43.60 cm/s

Estimated renal oxygen delivery = 38.93 arbitrary units (au)

HWI

Baseline

Baseline HbCO = 1.28%

(100 – 1.28)/100 = 0.9872

Renal blood velocity = 47.67 cm/s

Estimated renal oxygen delivery = 47.05 arbitrary units (au)

Post-HWI

Unchanged HbCO = 1.28%

(100 – 1.28)/100 = 0.9872

Minimum renal blood velocity = 35.90 cm/s

Estimated renal oxygen delivery = 35.44 arbitrary units (au)

COi + HWI

Baseline

Baseline HbCO = 1.28%

(100 – 1.28)/100 = 0.9872

Renal blood velocity = 42.97 cm/s

Estimated renal oxygen delivery = 42.42 arbitrary units (au)

Post-COi + HWI

Peak HbCO = 10.7%

(100 – 10.7)/100 = 0.8930

Minimum renal blood velocity = 30.10 cm/s

Estimated renal oxygen delivery = 26.88 arbitrary units (au)

Calculation 2: Hb mass

Participant data

Height: 180.3cm

Weight: 78.7kg

Age: 19

Sex: M

Volume of CO administered: 80 mL

Barometric pressure (P_{bar}): 749.5mmHg

Room temperature (test 1): 22.9˚C

Room temperature (test 2): 22.8˚C

Volume of gas remaining in circuit (test 1): 4000mL

Volume of gas remaining in circuit (test 2): 3800mL

ppm of gas remaining in circuit (test 1): 250

ppm of gas remaining in circuit (test 2): 420

Change in HbCO (test 1): 5.30%

Change in HbCO (test 2): 5.23%

Deadspace of circuit: 1242 mL

Equations used:

$P_{atm} = P_{bar} \bullet 0.0013157896611399$

Room temperature (K) = room temperature (C) + 273.15

Residual volume (mL) = (0.0275 • age) + (0.0189 • height (cm)) – 2.6139

Volume of CO unabsorbed (mL) = (ppm remaining in circuit • ((volume of gas remaining in circuit (mL) + deadspace of circuit (mL)) + (residual volume (mL)) ÷ 1000000)

Volume of CO absorbed (L) = [volume of CO administered (mL) – volume of CO unabsorbed (mL)] ÷ 1000

nCOabsorbed (moles) = [P_{atm} • Volume of CO absorbed (L)] ÷ [0.08206 • room temperature (K)]

nHbtagged (moles) = nCOabsorbed (moles) ÷ 4

nHbtotal (moles) = (nHbtagged (moles) ÷ ΔHbCO) • 100

Hb mass (g) = nHbtotal (moles) • $6.44 \bullet 10^4$

Calculation of Hb mass (test 1):

P_{atm} = 749.5 • 0.0013157896611399 = 0.986

Room temperature (K) = 22.9 + 273.15 = 296.05

Residual volume (mL) = (0.0275 • 19) + (0.0189 • 180.3) – 2.6139 = 1.3163

Volume of CO unabsorbed (mL) = (250 • ((4000 + 1242) + (1.3163)) ÷ 1000000) = 1.6396

Volume of CO absorbed (L) = [80 – 1.6396] ÷ 1000 = 0.0784

nCOabsorbed (moles) = [0.986 • 0.0784] ÷ [0.08206 • 296.05] = 0.003180961

nHbtagged (moles) = 0.003180961 ÷ 4 = 0.00079524

nHbtotal (moles) = (0.00079524 ÷ 5.30) • 100 = 0.0150

Hb mass (g) = 0.0150 • $6.44 \bullet 10^4$ = **966.29**

Calculation of Hb mass (test 2):

P_{atm} = 749.5 • 0.0013157896611399 = 0.986

Room temperature (K) = 22.8 + 273.15 = 295.95

Residual volume (mL) = (0.0275 • 19) + (0.0189 • 180.3) – 2.6139 = 1.3163

Volume of CO unabsorbed (mL) = (420 • ((3800 + 1242)) + (1.3163)) ÷ 1000000) = 2.6704

Volume of CO absorbed (L) = [80 – 2.6704] ÷ 1000 = 0.0773

nCOabsorbed (moles) = [0.986 • 0.0773] ÷ [0.08206 • 295.95] = 0.003140173

nHbtagged (moles) = 0.003140173 ÷ 4 = 0.000785043

nHbtotal (moles) = (0.000785043 ÷ 5.23) • 100 = 0.0150

Hb mass (g) = 0.0150 • 6.44•10^4 = **966.05**

Calculation of percent error:

Mean Hb mass = 966.17

Standard deviation (StDev) = 0.1688

StDev/square root of 2 = 0.1193

Percent error = [(StDev/square root of 2) ÷ mean] • 100 = 0.1193 ÷ 966.17 = 0.0124%

CHAPTER IX

REFERENCES CITED

Aaronson PI, Robertson TP & Ward JPT (2002). Endothelium-derived mediators and hypoxic pulmonary vasoconstriction. *Respir Physiol Neurobiol* **132,** 107–120.

Abbrecht PH & Littell JK (1972). Plasma erythropoietin in men and mice during acclimatization to different altitudes. *J Appl Physiol* **32,** 54–58.

Abreu R, Essler L, Giri P & Quinn F (2020). Interferon-gamma promotes iron export in human macrophages to limit intracellular bacterial replication. *PLOS ONE* **15,** e0240949.

Akerman AP, Lucas SJE, Katare R & Cotter JD (2017). Heat and Dehydration Additively Enhance Cardiovascular Outcomes following Orthostatically-Stressful Calisthenics Exercise. *Front Physiol* **8,** 756.

Allemann Y, Hutter D, Lipp E, Sartori C, Duplain H, Egli M, Cook S, Scherrer U & Seiler C (2006). Patent foramen ovale and high-altitude pulmonary edema. *JAMA* **296,** 2954–2958.

Alvarez-Hernández X, Licéaga J, McKay IC & Brock JH (1989). Induction of hypoferremia and modulation of macrophage iron metabolism by tumor necrosis factor. *Lab Invest* **61,** 319–322.

Archer SL, Huang J, Henry T, Peterson D & Weir EK (1993). A redox-based O2 sensor in rat pulmonary vasculature. *Circ Res* **73,** 1100–1112.

Archer SL, London B, Hampl V, Wu X, Nsair A, Puttagunta L, Hashimoto K, Waite RE & Michelakis ED (2001). Impairment of hypoxic pulmonary vasoconstriction in mice lacking the voltage-gated potassium channel Kv1.5. *The FASEB Journal* **15,** 1801–1803.

Arend WP, Palmer G & Gabay C (2008). IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev* **223,** 20–38.

Bachman E, Travison TG, Basaria S, Davda MN, Guo W, Li M, Connor Westfall J, Bae H, Gordeuk V & Bhasin S (2014). Testosterone induces erythrocytosis via increased erythropoietin and suppressed hepcidin: evidence for a new erythropoietin/hemoglobin set point. *J Gerontol A Biol Sci Med Sci* **69,** 725–735.

Bailey DM, Dehnert C, Luks AM, Menold E, Castell C, Schendler G, Faoro V, Gutowski M, Evans KA, Taudorf S, James PE, McEneny J, Young IS, Swenson ER, Mairbäurl H, Bärtsch P & Berger MM (2010). High-altitude pulmonary hypertension is associated with a free radical-mediated reduction in pulmonary nitric oxide bioavailability. *J Physiol* **588,** 4837–4847.

Bain BJ (1996). Ethnic and sex differences in the total and differential white cell count and platelet count. *J Clin Pathol* **49,** 664–666.

Bain BJ & England JM (1975). Normal haematological values: sex difference in neutrophil count. *Br Med J* **1,** 306–309.

Bamira D & Picard MH (2018). Imaging: Echocardiology—Assessment of Cardiac Structure and Function. In *Encyclopedia of Cardiovascular Research and Medicine*, ed. Vasan RS & Sawyer DB, pp. 35–54. Elsevier, Oxford. Available at: https://www.sciencedirect.com/science/article/pii/B9780128096574109536 [Accessed May 1, 2023].

Banzet S, Sanchez H, Chapot R, Bigard X, Vaulont S & Koulmann N (2012). Interleukin-6 contributes to hepcidin mRNA increase in response to exercise. *Cytokine* **58,** 158–161.

Baranauskas MN, Fulton TJ, Fly AD, Martin BJ, Mickleborough TD & Chapman RF (2022). High Intraindividual Variability in the Response of Serum Erythropoietin to Multiple Simulated Altitude Exposures. *High Alt Med Biol* **23,** 85–89.

Barnes H, Brown Z, Burns A & Williams T (2019). Phosphodiesterase 5 inhibitors for pulmonary hypertension. *Cochrane Database Syst Rev* **1,** CD012621.

Bedke T, Muscate F, Soukou S, Gagliani N & Huber S (2019). IL-10-producing T cells and their dual functions. *Seminars in Immunology* **44,** 101335.

Berglund B, Gennser M, Ornhagen H, Ostberg C & Wide L (2002). Erythropoietin concentrations during 10 days of normobaric hypoxia under controlled environmental circumstances. *Acta Physiol Scand* **174,** 225–229.

Bhoopalan SV, Huang LJ & Weiss MJ (2020). Erythropoietin regulation of red blood cell production: from bench to bedside and back. ; DOI: 10.12688/f1000research.26648.1. Available at: https://f1000research.com/articles/9-1153 [Accessed February 14, 2022].

Bigham AW (2016). Genetics of human origin and evolution: high-altitude adaptations. *Current Opinion in Genetics & Development* **41,** 8–13.

Blitzer ML, Loh E, Roddy M-A, Stamler JS & Creager MA (1996). Endothelium-derived nitric oxide regulates systemic and pulmonary vascular resistance during acute hypoxia in humans. *Journal of the American College of Cardiology* **28,** 591–596.

Blobel GA & Orkin SH (1996). Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. *Mol Cell Biol* **16,** 1687–1694.

Boer P (1984). Estimated lean body mass as an index for normalization of body fluid volumes in humans. *Am J Physiol* **247,** F632-636.

Boutin AT, Weidemann A, Fu Z, Mesropian L, Gradin K, Jamora C, Wiesener M, Eckardt K-U, Koch CJ, Ellies LG, Haddad G, Haase VH, Simon MC, Poellinger L, Powell FL & Johnson RS (2008). Epidermal sensing of oxygen is essential for systemic hypoxic response. *Cell* **133,** 223–234.

Bradbury KE, Coffman KE, Mitchell KM, Luippold AJ, Fulco CS & Kenefick RW (2019). Separate and combined influences of heat and hypobaric hypoxia on self-paced aerobic exercise performance. *Journal of Applied Physiology* **127,** 513–519.

Broudy VC, Lin N, Brice M, Nakamoto B & Papayannopoulou T (1991). Erythropoietin receptor characteristics on primary human erythroid cells. *Blood* **77,** 2583–2590.

Broxmeyer HE, Lu L, Platzer E, Feit C, Juliano L & Rubin BY (1983). Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. *The Journal of Immunology* **131,** 1300–1305.

Brubakk AO, Duplancic D, Valic Z, Palada I, Obad A, Bakovic D, Wisloff U & Dujic Z (2005). A single air dive reduces arterial endothelial function in man. *J Physiol* **566,** 901–906.

Buchheit M, Voss SC, Nybo L, Mohr M & Racinais S (2011). Physiological and performance adaptations to an in-season soccer camp in the heat: associations with heart rate and heart rate variability. *Scand J Med Sci Sports* **21,** e477-485.

Cappellini MD, Musallam KM & Taher AT (2020). Iron deficiency anaemia revisited. *Journal of Internal Medicine* **287,** 153–170.

Carter EA, Lohse K, Sheel W & Koehle M (2019). Sildenafil does not reliably improve exercise performance in hypoxia: a systematic review. *BMJ Open Sport Exerc Med* **5,** e000526.

Chao J, Donham P, van Rooijen N, Wood JG & Gonzalez NC (2011). Monocyte Chemoattractant Protein–1 Released from Alveolar Macrophages Mediates the Systemic Inflammation of Acute Alveolar Hypoxia. *Am J Respir Cell Mol Biol* **45,** 53–61.

Chapman CL, Holt SM, O'Connell CT, Brazelton SC, Howells WAB, Medved HN, Reed EL, Needham KW, Halliwill JR & Minson CT (2023). Acute Kidney Injury Biomarkers and Hydration Assessments Following Prolonged Mild Hypohydration in Healthy Young Adults. *Am J Physiol Renal Physiol*; DOI: 10.1152/ajprenal.00086.2023.

Chapman CL, Johnson BD, Hostler D, Lema PC & Schlader ZJ (2020). Reliability and agreement of human renal and segmental artery hemodynamics measured using Doppler ultrasound. *J Appl Physiol (1985)* **128,** 627–636.

Chen Y, Zhang Y, Zhao G, Chen C, Yang P, Ye S & Tan X (2016). Difference in Leukocyte Composition between Women before and after Menopausal Age, and Distinct Sexual Dimorphism. *PLOS ONE* **11,** e0162953.

Conboy EE, Fogelman AE, Sauder CL & Ray CA (2010). Endurance training reduces renal vasoconstriction to orthostatic stress. *American Journal of Physiology-Renal Physiology* **298,** F279–F284.

Cooper AC, Mikhail A, Lethbridge MW, Kemeny DM & Macdougall IC (2003). Increased expression of erythropoiesis inhibiting cytokines (IFN-gamma, TNF-alpha, IL-10, and IL-13) by T cells in patients exhibiting a poor response to erythropoietin therapy. *J Am Soc Nephrol* **14,** 1776–1784.

Cooper CJ, Landzberg MJ, Anderson TJ, Charbonneau F, Creager MA, Ganz P & Selwyn AP (1996). Role of Nitric Oxide in the Local Regulation of Pulmonary Vascular Resistance in Humans. *Circulation* **93,** 266–271.

Coronado Daza JA & Cuchi GU (2019). Gender Differences in Dose of Erythropoietin to Maintain Hemoglobin Target in Hemodialysis Patients. *Indian J Nephrol* **29,** 160–165.

Corre I, Pineau D & Hermouet S (1999). Interleukin-8: an autocrine/paracrine growth factor for human hematopoietic progenitors acting in synergy with colony stimulating factor-1 to promote monocyte-macrophage growth and differentiation. *Exp Hematol* **27,** 28–36.

Culic VC, Van Craenenbroeck E, Muzinic NR, Ljubkovic M, Marinovic J, Conraads V & Dujic Z (2014). Effects of scuba diving on vascular repair mechanisms. *Undersea Hyperb Med* **41,** 97–104.

Dai CH, Price JO, Brunner T & Krantz SB (1998). Fas ligand is present in human erythroid colony-forming cells and interacts with Fas induced by interferon gamma to produce erythroid cell apoptosis. *Blood* **91,** 1235–1242.

Dalton J (1826). On the Constitution of the Atmosphere. *Philosophical Transactions of the Royal Society of London* **116,** 174–188.

Davis JT, Hay MW, Hardin AM, White MD & Lovering AT (2017). Effect of a patent foramen ovale in humans on thermal responses to passive cooling and heating. *J Appl Physiol (1985)* **123,** 1423–1432.

Davis JT, Ng C-YA, Hill SD, Padgett RC & Lovering AT (2015). Higher oesophageal temperature at rest and during exercise in humans with patent foramen ovale. *J Physiol (Lond)* **593,** 4615–4630.

Desai A, Zhao Y, Lankford HA & Warren JS (2006). Nitric oxide suppresses EPO-induced monocyte chemoattractant protein-1 in endothelial cells: implications for atherogenesis in chronic renal disease. *Lab Invest* **86,** 369–379.

DiMarco KG, Beasley KM, Shah K, Speros JP, Elliott JE, Laurie SS, Duke JW, Goodman RD, Futral JE, Hawn JA, Roach RC & Lovering AT (2021). No effect of patent foramen ovale on acute

mountain sickness and pulmonary pressure in normobaric hypoxia. *Exp Physiol*; DOI: 10.1113/EP089948.

Dolznig H, Habermann B, Stangl K, Deiner EM, Moriggl R, Beug H & Müllner EW (2002). Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. *Curr Biol* **12,** 1076–1085.

Dominelli PB, Baker SE, Wiggins CC, Stewart GM, Sajgalik P, Shepherd JRA, Roberts SK, Roy TK, Curry TB, Hoyer JD, Oliveira JL, Foster GE & Joyner MJ (2019). Dissociating the effects of oxygen pressure and content on the control of breathing and acute hypoxic response. *J Appl Physiol (1985)* **127,** 1622–1631.

Dujic Z, Bakovic D, Marinovic-Terzic I & Eterovic D (2005*a*). Acute effects of a single open sea air dive and post-dive posture on cardiac output and pulmonary gas exchange in recreational divers. *Br J Sports Med* **39,** e24.

Dujić Z, Obad A, Palada I, Valic Z & Brubakk AO (2006). A single open sea air dive increases pulmonary artery pressure and reduces right ventricular function in professional divers. *Eur J Appl Physiol* **97,** 478–485.

Dujic Ž, Palada I, Obad A, Duplancic D, Bakovic D & Valic Z (2005*b*). Exercise during a 3-Min Decompression Stop Reduces Postdive Venous Gas Bubbles. *Medicine & Science in Sports & Exercise* **37,** 1319.

Duke JW, Beasley KM, Speros JP, Elliott JE, Laurie SS, Goodman RD, Futral E, Hawn JA & Lovering AT (2020). Impaired pulmonary gas exchange efficiency, but normal pulmonary artery pressure increases, with hypoxia in men and women with a patent foramen ovale. *Exp Physiol*; DOI: 10.1113/EP088750.

Duke JW, Davis JT, Ryan BJ, Elliott JE, Beasley KM, Hawn JA, Byrnes WC & Lovering AT (2016). Decreased arterial PO2, not O2 content, increases blood flow through intrapulmonary arteriovenous anastomoses at rest. *J Physiol* **594,** 4981–4996.

Duke JW, Elliott JE, Laurie SS, Beasley KM, Mangum TS, Hawn JA, Gladstone IM & Lovering AT (2014). Pulmonary gas exchange efficiency during exercise breathing normoxic and hypoxic gas in adults born very preterm with low diffusion capacity. *J Appl Physiol (1985)* **117,** 473–481.

Dukes PP & Goldwasser E (1961). Inhibition of erythropoiesis by estrogens. *Endocrinology* **69,** 21–29.

Dunham-Snary KJ, Wu D, Sykes EA, Thakrar A, Parlow LRG, Mewburn JD, Parlow JL & Archer SL (2017). Hypoxic Pulmonary Vasoconstriction: From Molecular Mechanisms to Medicine. *Chest* **151,** 181–192.

Dybedal I, Larsen S & Jacobsen SE (1995). IL-12 directly enhances in vitro murine erythropoiesis in combination with IL-4 and stem cell factor. *J Immunol* **154,** 4950–4955.

Dzierzak E & Philipsen S (2013). Erythropoiesis: Development and Differentiation. *Cold Spring Harb Perspect Med* **3,** a011601.

Eftedal I, Ljubkovic M, Flatberg A, Jørgensen A, Brubakk AO & Dujic Z (2013). Acute and potentially persistent effects of scuba diving on the blood transcriptome of experienced divers. *Physiol Genomics* **45,** 965–972.

Elliott GC, Gurtu R, McCollum C, Newman WG & Wang T (2014*a*). Foramen ovale closure is a process of endothelial-to-mesenchymal transition leading to fibrosis. *PLoS One* **9,** e107175.

Elliott JE, Duke JW, Hawn JA, Halliwill JR & Lovering AT (2014*b*). Increased cardiac output, not pulmonary artery systolic pressure, increases intrapulmonary shunt in healthy humans breathing room air and 40% O2. *J Physiol* **592,** 4537–4553.

Elliott JE, Nigam SM, Laurie SS, Beasley KM, Goodman RD, Hawn JA, Gladstone IM, Chesnutt MS & Lovering AT (2013). Prevalence of left heart contrast in healthy, young, asymptomatic humans at rest breathing room air. *Respir Physiol Neurobiol* **188,** 71–78.

Eng VM, Car BD, Schnyder B, Lorenz M, Lugli S, Aguet M, Anderson TD, Ryffel B & Quesniaux VF (1995). The stimulatory effects of interleukin (IL)-12 on hematopoiesis are antagonized by IL-12-induced interferon gamma in vivo. *J Exp Med* **181,** 1893–1898.

Etzrodt M, Ahmed N, Hoppe PS, Loeffler D, Skylaki S, Hilsenbeck O, Kokkaliaris KD, Kaltenbach H-M, Stelling J, Nerlov C & Schroeder T (2019). Inflammatory signals directly instruct PU.1 in HSCs via TNF. *Blood* **133,** 816–819.

Euler US v. & Liljestrand G (1946). Observations on the Pulmonary Arterial Blood Pressure in the Cat. *Acta Physiologica Scandinavica* **12,** 301–320.

Falz R, Fikenzer S, Hoppe S & Busse M (2019). Normal Values of Hemoglobin Mass and Blood Volume in Young, Active Women and Men. *Int J Sports Med* **40,** 236–244.

Fandrey J & Jelkmann WE (1991). Interleukin-1 and tumor necrosis factor-alpha inhibit erythropoietin production in vitro. *Ann N Y Acad Sci* **628,** 250–255.

Faquin WC, Schneider TJ & Goldberg MA (1992). Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. *Blood* **79,** 1987–1994.

Faura J, Ramos J, Reynafarje C, English E, Finne P & Finch CA (1969). Effect of Altitude on Erythropoiesis. *Blood* **33,** 668–676.

Felli N, Pedini F, Zeuner A, Petrucci E, Testa U, Conticello C, Biffoni M, Di Cataldo A, Winkles JA, Peschle C & De Maria R (2005). Multiple members of the TNF superfamily contribute to IFN-gamma-mediated inhibition of erythropoiesis. *J Immunol* **175,** 1464–1472.

Fenster BE, Curran-Everett D, Freeman AM, Weinberger HD, Kern Buckner J & Carroll JD (2014). Saline contrast echocardiography for the detection of patent foramen ovale in hypoxia: a validation study using intracardiac echocardiography. *Echocardiography* **31,** 420–427.

Foster GE, Ainslie PN, Stembridge M, Day TA, Bakker A, Lucas SJE, Lewis NCS, MacLeod DB & Lovering AT (2014). Resting pulmonary haemodynamics and shunting: a comparison of sea-level inhabitants to high altitude Sherpas. *J Physiol* **592,** 1397–1409.

Fraenkel PG (2017). Anemia of Inflammation: A Review. *Med Clin North Am* **101,** 285–296.

Friedmann B, Frese F, Menold E, Kauper F, Jost J & Bärtsch P (2005). Individual variation in the erythropoietic response to altitude training in elite junior swimmers. *British Journal of Sports Medicine* **39,** 148–153.

Fujita M, Mason RJ, Cool C, Shannon JM, Hara N & Fagan KA (2002). Pulmonary hypertension in TNF-α-overexpressing mice is associated with decreased VEGF gene expression. *Journal of Applied Physiology* **93,** 2162–2170.

Gaffen SL (2009). Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* **9,** 556–567.

Ganz T (2007). Molecular control of iron transport. *J Am Soc Nephrol* **18,** 394–400.

Ganz T (2016). Hepcidin. *Rinsho Ketsueki* **57,** 1913–1917.

Ganz T (2019). Anemia of Inflammation. *N Engl J Med* **381,** 1148–1157.

Garvican L, Martin D, Quod M, Stephens B, Sassi A & Gore C (2012). Time course of the hemoglobin mass response to natural altitude training in elite endurance cyclists. *Scand J Med Sci Sports* **22,** 95–103.

GBD 2016 Disease and Injury Incidence and Prevalence Collaborators (2017). Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet* **390,** 1211–1259.

Ge R-L, Witkowski S, Zhang Y, Alfrey C, Sivieri M, Karlsen T, Resaland GK, Harber M, Stray-Gundersen J & Levine BD (2002). Determinants of erythropoietin release in response to short-term hypobaric hypoxia. *Journal of Applied Physiology* **92,** 2361–2367.

Geissler K, Ohler L, Födinger M, Kabrna E, Kollars M, Skoupy S & Lechner K (1998). Interleukin-10 inhibits erythropoietin-independent growth of erythroid bursts in patients with polycythemia vera. *Blood* **92,** 1967–1972.

George PM et al. (2014). Evidence for the involvement of type I interferon in pulmonary arterial hypertension. *Circ Res* **114,** 677–688.

Gillinder KR, Tuckey H, Bell CC, Magor GW, Huang S, Ilsley MD & Perkins AC (2017). Direct targets of pSTAT5 signalling in erythropoiesis. *PLoS One* **12,** e0180922.

Goebel U, Siepe M, Mecklenburg A, Stein P, Roesslein M, Schwer CI, Schmidt R, Doenst T, Geiger KK, Pahl HL, Schlensak C & Loop T (2008). Carbon monoxide inhalation reduces pulmonary inflammatory response during cardiopulmonary bypass in pigs. *Anesthesiology* **108,** 1025–1036.

Goebel U & Wollborn J (2020). Carbon monoxide in intensive care medicine-time to start the therapeutic application?! *Intensive Care Med Exp* **8,** 2.

Goodrich JA, Frisco DJ, Kim S, Holliday M, Rueda M, Poddar S & Byrnes WC (2020). The importance of lean mass and iron deficiency when comparing hemoglobin mass in male and female athletic groups. *Journal of Applied Physiology* **129,** 855–863.

Goodrich JA, Ryan BJ & Byrnes WC (2018). The Influence of Oxygen Saturation on the Relationship Between Hemoglobin Mass and VO 2 max. *Sports Med Int Open* **2,** E98–E104.

Goss KN, Beshish AG, Barton GP, Haraldsdottir K, Levin TS, Tetri LH, Battiola TJ, Mulchrone AM, Pegelow DF, Palta M, Lamers LJ, Watson AM, Chesler NC & Eldridge MW (2018). Early Pulmonary Vascular Disease in Young Adults Born Preterm. *Am J Respir Crit Care Med* **198,** 1549–1558.

Govus AD, Garvican-Lewis LA, Abbiss CR, Peeling P & Gore CJ (2015). Pre-Altitude Serum Ferritin Levels and Daily Oral Iron Supplement Dose Mediate Iron Parameter and Hemoglobin Mass Responses to Altitude Exposure. *PLoS One* **10,** e0135120.

Grebien F, Kerenyi MA, Kovacic B, Kolbe T, Becker V, Dolznig H, Pfeffer K, Klingmüller U, Müller M, Beug H, Müllner EW & Moriggl R (2008). Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2. *Blood* **111,** 4511–4522.

Groth A, Vrugt B, Brock M, Speich R, Ulrich S & Huber LC (2014). Inflammatory cytokines in pulmonary hypertension. *Respir Res* **15,** 47.

Groves BM, Reeves JT, Sutton JR, Wagner PD, Cymerman A, Malconian MK, Rock PB, Young PM & Houston CS (1987). Operation Everest II: elevated high-altitude pulmonary resistance unresponsive to oxygen. *J Appl Physiol (1985)* **63,** 521–530.

Grünig E, Mereles D, Hildebrandt W, Swenson ER, Kübler W, Kuecherer H & Bärtsch P (2000). Stress Doppler echocardiography for identification of susceptibility to high altitude pulmonary edema. *J Am Coll Cardiol* **35,** 980–987.

Haase VH (2010). Hypoxic regulation of erythropoiesis and iron metabolism. *Am J Physiol Renal Physiol* **299,** F1-13.

Haase VH (2013). Regulation of erythropoiesis by hypoxia-inducible factors. *Blood Rev* **27,** 41–53.

Hagan RD, Diaz FJ & Horvath SM (1978). Plasma volume changes with movement to supine and standing positions. *Journal of Applied Physiology* **45,** 414–417.

Hagen PT, Scholz DG & Edwards WD (1984). Incidence and size of patent foramen ovale during the first 10 decades of life: an autopsy study of 965 normal hearts. *Mayo Clin Proc* **59,** 17–20.

Handelsman DJ, Hirschberg AL & Bermon S (2018). Circulating Testosterone as the Hormonal Basis of Sex Differences in Athletic Performance. *Endocr Rev* **39,** 803–829.

Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N & Matsushima K (1994). Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* **56,** 559–564.

Hasunuma K, Rodman DM & McMurtry IF (1991). Effects of K+ channel blockers on vascular tone in the perfused rat lung. *Am Rev Respir Dis* **144,** 884–887.

Hayashi N, Yatsutani H, Mori H, Ito H, Badenhorst CE & Goto K (2020). No effect of supplemented heat stress during an acute endurance exercise session in hypoxia on hepcidin regulation. *Eur J Appl Physiol* **120,** 1331–1340.

van der Heijden T, Bot I & Kuiper J (2019). The IL-12 cytokine family in cardiovascular diseases. *Cytokine* **122,** 154188.

Hermouet S, Godard A, Pineau D, Corre I, Raher S, Lippert E & Jacques Y (2002). ABNORMAL PRODUCTION OF INTERLEUKIN (IL)-11 AND IL-8 IN POLYCYTHAEMIA VERA. *Cytokine* **20,** 178–183.

Hess DR (2017). Inhaled Carbon Monoxide: From Toxin to Therapy. *Respir Care* **62,** 1333–1342.

Himelman RB, Stulbarg M, Kircher B, Lee E, Kee L, Dean NC, Golden J, Wolfe CL & Schiller NB (1989*a*). Noninvasive evaluation of pulmonary artery pressure during exercise by saline-enhanced Doppler echocardiography in chronic pulmonary disease. *Circulation* **79,** 863–871.

Himelman RB, Stulbarg M, Kircher B, Lee E, Kee L, Dean NC, Golden J, Wolfe CL & Schiller NB (1989*b*). Noninvasive evaluation of pulmonary artery pressure during exercise by saline-

enhanced Doppler echocardiography in chronic pulmonary disease. *Circulation* **79,** 863–871.

Hlastala MP, Lamm WJE, Karp A, Polissar NL, Starr IR & Glenny RW (2004). Spatial distribution of hypoxic pulmonary vasoconstriction in the supine pig. *J Appl Physiol (1985)* **96,** 1589–1599.

Hoffman JIE (2011). Pulmonary vascular resistance and viscosity: the forgotten factor. *Pediatr Cardiol* **32,** 557–561.

Hopkins SR, Belzberg AS, Wiggs BR & McKenzie DC (1996). Pulmonary transit time and diffusion limitation during heavy exercise in athletes. *Respir Physiol* **103,** 67–73.

Huang P, Wang J, Lin X, Yang F-F & Tan J-H (2017). Effects of IL-10 on iron metabolism in LPS-induced inflammatory mice via modulating hepcidin expression. *Eur Rev Med Pharmacol Sci* **21,** 3469–3475.

Hwang Y, Hidalgo D, Khoramian Tusi B, Wolock S, Weinreb C, Klein AM & Socolovsky M (2017). The IL-17 Receptor IL-17RA Is Expressed By Early Erythroid Progenitors and Stimulates Erythropoiesis. *Blood* **130,** 3467.

Ito T, Okada T, Miyashita H, Nomoto T, Nonaka-Sarukawa M, Uchibori R, Maeda Y, Urabe M, Mizukami H, Kume A, Takahashi M, Ikeda U, Shimada K & Ozawa K (2007). Interleukin-10 Expression Mediated by an Adeno-Associated Virus Vector Prevents Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats. *Circulation Research* **101,** 734–741.

Jacobsen SE, Veiby OP & Smeland EB (1993). Cytotoxic lymphocyte maturation factor (interleukin 12) is a synergistic growth factor for hematopoietic stem cells. *J Exp Med* **178,** 413–418.

Jelkmann W (1998). Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res* **18,** 555–559.

Jelkmann W (2011). Regulation of erythropoietin production. *J Physiol* **589,** 1251–1258.

Kalantar-Zadeh K (2017). History of Erythropoiesis-Stimulating Agents, the Development of Biosimilars, and the Future of Anemia Treatment in Nephrology. *Am J Nephrol* **45,** 235–247.

Kaufman JS (2008). Racial Differences in Erythropoietin Responsiveness. *American Journal of Kidney Diseases* **52,** 1035–1038.

Keiser S, Siebenmann C, Bonne TC, Sørensen H, Robach P & Lundby C (2013). The carbon monoxide re-breathing method can underestimate Hbmass due to incomplete blood mixing. *Eur J Appl Physiol* **113,** 2425–2430.

Keithi-Reddy SR, Addabbo F, Patel TV, Mittal BV, Goligorsky MS & Singh AK (2008). Association of anemia and erythropoiesis stimulating agents with inflammatory biomarkers in chronic kidney disease. *Kidney Int* **74,** 782–790.

Keller MF, Harrison ML & Lalande S (2020). Impact of Menstrual Blood Loss and Oral Contraceptive Use on Oxygen-carrying Capacity. *Med Sci Sports Exerc* **52,** 1414–1419.

Kelly T, Brown C, Bryant-Ekstrand M, Lord R, Dawkins T, Drane A, Futral JE, Barak O, Dragun T, Stembridge M, Spajić B, Drviš I, Duke JW, Ainslie PN, Foster GE, Dujic Z & Lovering AT (2022). Blunted hypoxic pulmonary vasoconstriction in apnoea divers. *Exp Physiol* **107,** 1225–1240.

Kimura H, Okada O, Tanabe N, Tanaka Y, Terai M, Takiguchi Y, Masuda M, Nakajima N, Hiroshima K, Inadera H, Matsushima K & Kuriyama T (2001). Plasma monocyte chemoattractant protein-1 and pulmonary vascular resistance in chronic thromboembolic pulmonary hypertension. *Am J Respir Crit Care Med* **164,** 319–324.

Kirby NV, Lucas SJE, Armstrong OJ, Weaver SR & Lucas RAI (2021). Intermittent post-exercise sauna bathing improves markers of exercise capacity in hot and temperate conditions in trained middle-distance runners. *Eur J Appl Physiol* **121,** 621–635.

Klausen T (1998). The feed-back regulation of erythropoietin production in healthy humans. *Dan Med Bull* **45,** 345–353.

Klein SL & Flanagan KL (2016). Sex differences in immune responses. *Nat Rev Immunol* **16,** 626–638.

Koivisto-Mørk AE, Svendsen IS, Skattebo Ø, Hallén J & Paulsen G (2021). Impact of baseline serum ferritin and supplemental iron on altitude-induced hemoglobin mass response in elite athletes. *Scand J Med Sci Sports* **31,** 1764–1773.

Kovacs G, Olschewski A, Berghold A & Olschewski H (2012). Pulmonary vascular resistances during exercise in normal subjects: a systematic review. *European Respiratory Journal* **39,** 319–328.

Kramers C, de Mulder PH, Barth JD & Wagener DJ (1993). Acute right ventricular heart failure in a patient with renal cell carcinoma after interferon therapy. *Neth J Med* **42,** 65–68.

Krstic A, Kocic J, Ilic V, Mojsilovic S, Okic-Dordevic I, Trivanovic D, Santibanez JF, Jovcic G & Bugarski D (2012). Effects of IL-17 on erythroid progenitors growth: involvement of MAPKs and GATA transcription factors. *J Biol Regul Homeost Agents* **26,** 641–652.

La Ferla K, Reimann C, Jelkmann W & Hellwig-Bürgel T (2002). Inhibition of erythropoietin gene expression signaling involves the transcription factors GATA-2 and NF-κB. *The FASEB Journal* **16,** 1–17.

Lacson E, Rogus J, Teng M, Lazarus JM & Hakim RM (2008). The association of race with erythropoietin dose in patients on long-term hemodialysis. *Am J Kidney Dis* **52,** 1104–1114.

Lai CM, Swaminathan N, Beilharz MW, Papadimitriou J & Klinken SP (1995). Interferon-alpha inhibits erythropoietin-induced proliferation, but not differentiation, and restricts erythroleukemia development. *J Interferon Cytokine Res* **15,** 669–675.

Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L, Flachskampf FA, Foster E, Goldstein SA, Kuznetsova T, Lancellotti P, Muraru D, Picard MH, Rietzschel ER, Rudski L, Spencer KT, Tsang W & Voigt J-U (2015). Recommendations for Cardiac Chamber Quantification by Echocardiography in Adults: An Update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Journal of the American Society of Echocardiography* **28,** 1-39.e14.

Laurie SS, Elliott JE, Beasley KM, Mangum TS, Goodman RD, Duke JW, Gladstone IM & Lovering AT (2018). Exaggerated Increase in Pulmonary Artery Pressure during Exercise in Adults Born Preterm. *Am J Respir Crit Care Med* **197,** 821–823.

Laurie SS, Elliott JE, Goodman RD & Lovering AT (2012). Catecholamine-induced opening of intrapulmonary arteriovenous anastomoses in healthy humans at rest. *J Appl Physiol (1985)* **113,** 1213–1222.

Laurie SS, Yang X, Elliott JE, Beasley KM & Lovering AT (2010). Hypoxia-induced intrapulmonary arteriovenous shunting at rest in healthy humans. *Journal of Applied Physiology* **109,** 1072–1079.

Levitsky MG (2007). *Pulmonary Physiology*, 7th edn. McGraw-Hill Medical.

Lew VL & Tiffert T (2017). On the Mechanism of Human Red Blood Cell Longevity: Roles of Calcium, the Sodium Pump, PIEZO1, and Gardos Channels. *Front Physiol* **8,** 977.

Libregts SF, Gutiérrez L, de Bruin AM, Wensveen FM, Papadopoulos P, van Ijcken W, Özgür Z, Philipsen S & Nolte MA (2011). Chronic IFN-γ production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU.1 axis. *Blood* **118,** 2578–2588.

Liu J, Wang W, Wang L, Chen S, Tian B, Huang K, Corrigan CJ, Ying S, Wang W & Wang C (2018*a*). IL-33 Initiates Vascular Remodelling in Hypoxic Pulmonary Hypertension by up-Regulating HIF-1α and VEGF Expression in Vascular Endothelial Cells. *EBioMedicine* **33,** 196–210.

Liu Y-S, Huang H, Zhou S-M, Tian H & Li P (2018*b*). Excessive Iron Availability Caused by Disorders of Interleukin-10 and Interleukin-22 Contributes to High Altitude Polycythemia. *Front Physiol* **9,** 548.

Lloyd A & Havenith G (2016). Interactions in human performance: An individual and combined stressors approach. *Temperature (Austin)* **3,** 514–517.

Lodish H, Flygare J & Chou S (2010). From stem cell to erythroblast: regulation of red cell production at multiple levels by multiple hormones. *IUBMB Life* **62,** 492–496.

López-Gómez JM, Portolés JM & Aljama P (2008). Factors that condition the response to erythropoietin in patients on hemodialysis and their relation to mortality. *Kidney International* **74,** S75–S81.

Lorenzo S, Halliwill JR, Sawka MN & Minson CT (2010). Heat acclimation improves exercise performance. *Journal of Applied Physiology* **109,** 1140–1147.

Lovering A & Goodman R (2012). Detection of Intracardiac and Intrapulmonary Shunts at Rest and During Exercise Using Saline Contrast Echocardiography. In *Applied Aspects of Ultrasonography in Humans*, ed. Ainslie P. InTech. Available at: http://www.intechopen.com/books/applied-aspects-of-ultrasonography-in-humans/detection-of-intracardiac-and-intrapulmonary-shunts-at-rest-and-during-exercise-and-stress-using-ech [Accessed September 9, 2020].

Lovering AT, Stickland MK, Amann M, O'Brien MJ, Hokanson JS & Eldridge MW (2011). Effect of a patent foramen ovale on pulmonary gas exchange efficiency at rest and during exercise. *J Appl Physiol (1985)* **110,** 1354–1361.

Lu L, Xiao M, Grigsby S, Wang WX, Wu B, Shen RN & Broxmeyer HE (1993). Comparative effects of suppressive cytokines on isolated single CD34(3+) stem/progenitor cells from human bone marrow and umbilical cord blood plated with and without serum. *Exp Hematol* **21,** 1442–1446.

Lu Y, Basatemur G, Scott IC, Chiarugi D, Clement M, Harrison J, Jugdaohsingh R, Yu X, Newland SA, Jolin HE, Li X, Chen X, Szymanska M, Haraldsen G, Palmer G, Fallon PG, Cohen ES, McKenzie ANJ & Mallat Z (2020). Interleukin-33 Signaling Controls the Development of Iron-Recycling Macrophages. *Immunity* **52,** 782-793.e5.

Lumb A (2005). *Nunns applied respiratory physiology*, 6th edn.

Lundby C, Hamarsland H, Hansen J, Bjørndal H, Berge SN, Hammarström D & Rønnestad BR (2023). Hematological, skeletal muscle fiber and exercise performance adaptations to heat training in elite female and male cyclists. *J Appl Physiol (1985)*; DOI: 10.1152/japplphysiol.00115.2023.

Lundby C, Thomsen JJ, Boushel R, Koskolou M, Warberg J, Calbet JAL & Robach P (2007). Erythropoietin treatment elevates haemoglobin concentration by increasing red cell volume and depressing plasma volume. *J Physiol* **578,** 309–314.

Madden D, Thom SR, Milovanova TN, Yang M, Bhopale VM, Ljubkovic M & Dujic Z (2014). Exercise before scuba diving ameliorates decompression-induced neutrophil activation. *Med Sci Sports Exerc* **46,** 1928–1935.

Madden JA, Vadula MS & Kurup VP (1992). Effects of hypoxia and other vasoactive agents on pulmonary and cerebral artery smooth muscle cells. *Am J Physiol* **263,** L384-393.

Madden LA, Chrismas BC, Mellor D, Vince RV, Midgley AW, McNaughton LR, Atkin SL & Laden G (2010). Endothelial function and stress response after simulated dives to 18 msw breathing air or oxygen. *Aviat Space Environ Med* **81,** 41–45.

Malik AB & Kidd BSL (1976). Pulmonary Arterial Wedge and Left Atrial Pressures and the Site of Hypoxic Pulmonary Vasoconstriction. *RES* **33,** 123–132.

Mamus SW, Beck-Schroeder S & Zanjani ED (1985). Suppression of normal human erythropoiesis by gamma interferon in vitro. Role of monocytes and T lymphocytes. *J Clin Invest* **75,** 1496–1503.

Marabotti C, Chiesa F, Scalzini A, Antonelli F, Lari R, Franchini C & Data PG (1999). Cardiac and humoral changes induced by recreational scuba diving. *Undersea Hyperb Med* **26,** 151–158.

Marabotti C, Scalzini A & Chiesa F (2013). Increase of pulmonary arterial pressure in subjects with venous gas emboli after uncomplicated recreational SCUBA diving. *Respir Med* **107,** 596–600.

Marriott K, Manins V, Forshaw A, Wright J & Pascoe R (2013). Detection of right-to-left atrial communication using agitated saline contrast imaging: experience with 1162 patients and recommendations for echocardiography. *J Am Soc Echocardiogr* **26,** 96–102.

Martina SD, Freiberger JJ, Peacher DF, Natoli MJ, Schinazi EA, Kernagis DN, Potter JVF, Otteni CE & Moon RE (2017). Sildenafil: Possible Prophylaxis against Swimming-induced Pulmonary Edema. *Med Sci Sports Exerc* **49,** 1755–1757.

McCleave EL, Slattery KM, Duffield R, Saunders PU, Sharma AP, Crowcroft SJ & Coutts AJ (2017). Temperate Performance Benefits after Heat, but Not Combined Heat and Hypoxic Training. *Med Sci Sports Exerc* **49,** 509–517.

McCranor BJ, Kim MJ, Cruz NM, Xue Q-L, Berger AE, Walston JD, Civin CI & Roy CN (2014). Interleukin-6 directly impairs the erythroid development of human TF-1 erythroleukemic cells. *Blood Cells Mol Dis* **52,** 126–133.

McMurtry IF, Davidson AB, Reeves JT & Grover RF (1976). Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs. *Circ Res* **38,** 99–104.

Melsom MN, Flatebø T & Nicolaysen G (1999). Hypoxia and hyperoxia both transiently affect distribution of pulmonary perfusion but not ventilation in awake sheep. *Acta Physiol Scand* **166,** 151–158.

Milledge JS & Cotes PM (1985). Serum erythropoietin in humans at high altitude and its relation to plasma renin. *J Appl Physiol (1985)* **59,** 360–364.

Miller MR et al. (2005). Standardisation of spirometry. *Eur Respir J* **26,** 319–338.

Mishra KP, Sharma N, Soree P, Gupta RK, Ganju L & Singh SB (2016). Hypoxia-Induced Inflammatory Chemokines in Subjects with a History of High-Altitude Pulmonary Edema. *Indian J Clin Biochem* **31,** 81–86.

Mitchell C, Rahko PS, Blauwet LA, Canaday B, Finstuen JA, Foster MC, Horton K, Ogunyankin KO, Palma RA & Velazquez EJ (2019). Guidelines for Performing a Comprehensive Transthoracic Echocardiographic Examination in Adults: Recommendations from the American Society of Echocardiography. *Journal of the American Society of Echocardiography* **32,** 1–64.

Miyake T, Kung CK & Goldwasser E (1977). Purification of human erythropoietin. *J Biol Chem* **252,** 5558–5564.

Mohan K, Sam H & Stevenson MM (1999). Therapy with a Combination of Low Doses of Interleukin 12 and Chloroquine Completely Cures Blood-Stage Malaria, Prevents Severe Anemia, and Induces Immunity to Reinfection. *Infect Immun* **67,** 513–519.

Mojsilović S, Jauković A, Santibañez JF & Bugarski D (2015). Interleukin-17 and Its Implication in the Regulation of Differentiation and Function of Hematopoietic and Mesenchymal Stem Cells. *Mediators Inflamm* **2015,** 470458.

Montero D & Lundby C (2019). Arterial oxygen content regulates plasma erythropoietin independent of arterial oxygen tension: a blinded crossover study. *Kidney Int* **95,** 173–177.

Moon RE, Cherry AD, Stolp BW & Camporesi EM (2009). Pulmonary gas exchange in diving. *Journal of Applied Physiology* **106,** 668–677.

Moon RE, Martina SD, Peacher DF, Potter JF, Wester TE, Cherry AD, Natoli MJ, Otteni CE, Kernagis DN, White WD & Freiberger JJ (2016). Swimming-Induced Pulmonary Edema: Pathophysiology and Risk Reduction With Sildenafil. *Circulation* **133,** 988–996.

Motley HL, Cournand A, Werko L, Himmelstein A & Dresdale D (1947). The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man. *American Journal of Physiology-Legacy Content* **150,** 315–320.

Muckenthaler MU, Spasic MV, Mleczko-Sanecka K, Zhu M, Pepperkok R, Runz H, Maus U, Gomes J, Pinto JP, Lawless M, Kulozik AE & Porto G (2011). A High Through-Put Screen Identifies MCP-1 (CCL2) As a Novel Regulator of Iron Homeostasis and a Modifier of Hereditary Hemochromatosis Disease Severity. *Blood* **118,** 685.

Murphy WG (2014). The sex difference in haemoglobin levels in adults - mechanisms, causes, and consequences. *Blood Rev* **28,** 41–47.

Nairz M, Sonnweber T, Schroll A, Theurl I & Weiss G (2012). The pleiotropic effects of erythropoietin in infection and inflammation. *Microbes Infect* **14,** 238–246.

Nanami M, Ookawara T, Otaki Y, Ito K, Moriguchi R, Miyagawa K, Hasuike Y, Izumi M, Eguchi H, Suzuki K & Nakanishi T (2005). Tumor Necrosis Factor-α–Induced Iron Sequestration and Oxidative Stress in Human Endothelial Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25,** 2495–2501.

Nandakumar SK, Ulirsch JC & Sankaran VG (2016). Advances in understanding erythropoiesis: evolving perspectives. *Br J Haematol* **173,** 206–218.

Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK & Ganz T (2004). IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* **113,** 1271–1276.

Ni S, Yuan Y, Kuang Y & Li X (2022). Iron Metabolism and Immune Regulation. *Frontiers in Immunology*. Available at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.816282 [Accessed January 18, 2023].

Norris HC, Mangum TS, Duke JW, Straley TB, Hawn JA, Goodman RD & Lovering AT (2014*a*). Exercise- and hypoxia-induced blood flow through intrapulmonary arteriovenous anastomoses is reduced in older adults. *J Appl Physiol (1985)* **116,** 1324–1333.

Norris HC, Mangum TS, Duke JW, Straley TB, Hawn JA, Goodman RD & Lovering AT (2014*b*). Exercise- and hypoxia-induced blood flow through intrapulmonary arteriovenous anastomoses is reduced in older adults. *J Appl Physiol (1985)* **116,** 1324–1333.

Nossum V, Hjelde A & Brubakk AO (2002). Small amounts of venous gas embolism cause delayed impairment of endothelial function and increase polymorphonuclear neutrophil infiltration. *Eur J Appl Physiol* **86,** 209–214.

Nossum V, Koteng S & Brubakk AO (1999). Endothelial damage by bubbles in the pulmonary artery of the pig. *Undersea Hyperb Med* **26,** 1–8.

Nummela A, Eronen T, Koponen A, Tikkanen H & Peltonen JE (2021). Variability in hemoglobin mass response to altitude training camps. *Scand J Med Sci Sports* **31,** 44–51.

Oberholzer L, Siebenmann C, Mikkelsen CJ, Junge N, Piil JF, Morris NB, Goetze JP, Meinild Lundby A-K, Nybo L & Lundby C (2019). Hematological Adaptations to Prolonged Heat Acclimation in Endurance-Trained Males. *Front Physiol* **10,** 1379.

O'Brien-Ladner AR, Nelson SR, Murphy WJ, Blumer BM & Wesselius LJ (2000). Iron Is a Regulatory Component of Human IL-1 β Production: Support for Regional Variability in the Lung. *Am J Respir Cell Mol Biol* **23,** 112–119.

Oehler L, Kollars M, Bohle B, Berer A, Reiter E, Lechner K & Geissler K (1999). Interleukin-10 inhibits burst-forming unit-erythroid growth by suppression of endogenous granulocyte-macrophage colony-stimulating factor production from T cells. *Experimental Hematology* **27,** 217–223.

Oertelt-Prigione S (2012). Immunology and the menstrual cycle. *Autoimmun Rev* **11,** A486-492.

Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y & Hattori K (1995). Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* **378,** 88–91.

Ong'echa JM, Remo AM, Kristoff J, Hittner JB, Were T, Ouma C, Otieno RO, Vulule JM, Keller CC, Awandare GA & Perkins DJ (2008). Increased Circulating Interleukin (IL)-23 in Children with Malarial Anemia. *Clin Immunol* **126,** 211–221.

Oparil S, Chen SJ, Meng QC, Elton TS, Yano M & Chen YF (1995). Endothelin-A receptor antagonist prevents acute hypoxia-induced pulmonary hypertension in the rat. *Am J Physiol* **268,** L95-100.

Owen JA, Punt J & Stranford SA (2013). *Kuby Immunology*, 7th edn. W. H. Freeman and Company.

Ozaki K & Leonard WJ (2002). Cytokine and Cytokine Receptor Pleiotropy and Redundancy. *Journal of Biological Chemistry* **277,** 29355–29358.

Papani R, Duarte AG, Lin Y, Kuo Y-F & Sharma G (2017). Pulmonary arterial hypertension associated with interferon therapy: a population-based study. *Multidiscip Respir Med* **12,** 1.

Parasuraman S, Walker S, Loudon BL, Gollop ND, Wilson AM, Lowery C & Frenneaux MP (2016). Assessment of pulmonary artery pressure by echocardiography—A comprehensive review. *Int J Cardiol Heart Vasc* **12,** 45–51.

Pastor-Fernández G, Mariblanca IR & Navarro MN (2020). Decoding IL-23 Signaling Cascade for New Therapeutic Opportunities. *Cells* **9,** 2044.

Paulson RF, Ruan B, Hao S & Chen Y (2020). Stress Erythropoiesis is a Key Inflammatory Response. *Cells* **9,** E634.

Pedersen BK, Steensberg A & Schjerling P (2001). Muscle-derived interleukin-6: possible biological effects. *J Physiol* **536,** 329–337.

Pennell LM, Galligan CL & Fish EN (2012). Sex affects immunity. *J Autoimmun* **38,** J282-291.

Perreault AA & Venters BJ (2018). Integrative view on how erythropoietin signaling controls transcription patterns in erythroid cells. *Curr Opin Hematol* **25,** 189–195.

Petersen AMW & Pedersen BK (2005*a*). The anti-inflammatory effect of exercise. *J Appl Physiol (1985)* **98,** 1154–1162.

Petersen AMW & Pedersen BK (2005*b*). The anti-inflammatory effect of exercise. *J Appl Physiol (1985)* **98,** 1154–1162.

Petousi N, Croft QPP, Cavalleri GL, Cheng H-Y, Formenti F, Ishida K, Lunn D, McCormack M, Shianna KV, Talbot NP, Ratcliffe PJ & Robbins PA (2014). Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *J Appl Physiol (1985)* **116,** 893–904.

Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk LV, Zhang S, Lakshminarasimhan R, Chin CP, Techner J-M, Will B, Nerlov C, Steidl U, Manz MG, Schroeder T & Passegué E (2016). Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. *Nat Cell Biol* **18,** 607–618.

Post JM, Hume JR, Archer SL & Weir EK (1992). Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am J Physiol* **262,** C882-890.

Prabhakar NR, Dinerman JL, Agani FH & Snyder SH (1995). Carbon monoxide: a role in carotid body chemoreception. *Proc Natl Acad Sci U S A* **92,** 1994–1997.

Prince OD, Langdon JM, Layman AJ, Prince IC, Sabogal M, Mak HH, Berger AE, Cheadle C, Chrest FJ, Yu Q, Andrews NC, Xue Q-L, Civin CI, Walston JD & Roy CN (2012). Late stage erythroid precursor production is impaired in mice with chronic inflammation. *Haematologica* **97,** 1648–1656.

Rabinovitch M, Guignabert C, Humbert M & Nicolls MR (2014). Inflammation and Immunity in the Pathogenesis of Pulmonary Arterial Hypertension. *Circ Res* **115,** 165–175.

Racinais S, Buchheit M, Bilsborough J, Bourdon PC, Cordy J & Coutts AJ (2014). Physiological and Performance Responses to a Training Camp in the Heat in Professional Australian Football Players. *International Journal of Sports Physiology and Performance* **9,** 598–603.

Raefsky EL, Platanias LC, Zoumbos NC & Young NS (1985). Studies of interferon as a regulator of hematopoietic cell proliferation. *The Journal of Immunology* **135,** 2507–2512.

Rocco M, Maggi L, Loffredo C, Pelli M, Benedetto PD, Fiorelli S, Simmaco M & De Blasi RA (2021). The impact of different gas mixtures on inflammatory responses in advanced recreational divers. *Diving Hyperb Med* **51,** 140–146.

Rønnestad BR, Hamarsland H, Hansen J, Holen E, Montero D, Whist JE & Lundby C (2021). Five weeks of heat training increases haemoglobin mass in elite cyclists. *Experimental Physiology* **106,** 316–327.

Rønnestad BR, Lid OM, Hansen J, Hamarsland H, Mølmen KS, Nygaard H, Ellefsen S, Hammarström D & Lundby C (2022). Heat suit training increases hemoglobin mass in elite cross-country skiers. *Scandinavian Journal of Medicine & Science in Sports* **32,** 1089–1098.

Ross DJ, Strieter RM, Fishbein MC, Ardehali A & Belperio JA (2012). Type I immune response cytokine-chemokine cascade is associated with pulmonary arterial hypertension. *J Heart Lung Transplant* **31,** 865–873.

Rothenberg EV, Hosokawa H & Ungerbäck J (2019). Mechanisms of Action of Hematopoietic Transcription Factor PU.1 in Initiation of T-Cell Development. *Frontiers in Immunology*. Available at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00228 [Accessed January 17, 2023].

Rowell LB (1993). *Human Cardiovascular Control*. Oxford University Press.

Rudski LG, Lai WW, Afilalo J, Hua L, Handschumacher MD, Chandrasekaran K, Solomon SD, Louie EK & Schiller NB (2010). Guidelines for the Echocardiographic Assessment of the Right Heart in Adults: A Report from the American Society of Echocardiography: Endorsed by the European Association of Echocardiography, a registered branch of the European Society of Cardiology, and the Canadian Society of Echocardiography. *Journal of the American Society of Echocardiography* **23,** 685–713.

Rutz S & Ouyang W (2016). Regulation of Interleukin-10 Expression. *Adv Exp Med Biol* **941,** 89–116.

Ryan BJ, Goodrich JA, Schmidt W, Kane LA & Byrnes WC (2016). Ten Days of Intermittent, Low-dose Carbon Monoxide Inhalation does not Significantly Alter Hemoglobin Mass, Aerobic Performance Predictors, or Peak-power Exercise Tolerance. *Int J Sports Med* **37,** 884–889.

Ryan BJ, Wachsmuth NB, Schmidt WF, Byrnes WC, Julian CG, Lovering AT, Subudhi AW & Roach RC (2014). AltitudeOmics: rapid hemoglobin mass alterations with early acclimatization to and de-acclimatization from 5260 m in healthy humans. *PLoS One* **9,** e108788.

Savale L, Chaumais M-C, O'Connell C, Humbert M & Sitbon O (2016). Interferon-induced pulmonary hypertension: an update. *Curr Opin Pulm Med* **22,** 415–420.

Savale L, Tu L, Rideau D, Izziki M, Maitre B, Adnot S & Eddahibi S (2009). Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. *Respir Res* **10,** 6.

Scaccabarozzi A, Arosio P, Weiss G, Valenti L, Dongiovanni P, Fracanzani AL, Mattioli M, Levi S, Fiorelli G & Fargion S (2000). Relationship between TNF-α and iron metabolism in differentiating human monocytic THP-1 cells. *British Journal of Haematology* **110,** 978–984.

Schmidt WFJ, Hoffmeister T, Haupt S, Schwenke D, Wachsmuth NB & Byrnes WC (2020). Chronic Exposure to Low-Dose Carbon Monoxide Alters Hemoglobin Mass and V˙O2max. *Med Sci Sports Exerc* **52,** 1879–1887.

Schooley JC, Kullgren B & Allison AC (1987). Inhibition by interleukin-1 of the action of erythropoietin on erythroid precursors and its possible role in the pathogenesis of hypoplastic anaemias. *Br J Haematol* **67,** 11–17.

Schumacker PT (2011). Lung cell hypoxia: role of mitochondrial reactive oxygen species signaling in triggering responses. *Proc Am Thorac Soc* **8,** 477–484.

Scoon GSM, Hopkins WG, Mayhew S & Cotter JD (2007). Effect of post-exercise sauna bathing on the endurance performance of competitive male runners. *J Sci Med Sport* **10,** 259–262.

Scott TE, Kemp-Harper BK & Hobbs AJ (2019). Inflammasomes: a novel therapeutic target in pulmonary hypertension? *British Journal of Pharmacology* **176,** 1880–1896.

Semenza GL, Nejfelt MK, Chi SM & Antonarakis SE (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proc Natl Acad Sci U S A* **88,** 5680–5684.

Shahani S, Braga-Basaria M, Maggio M & Basaria S (2009). Androgens and erythropoiesis: past and present. *J Endocrinol Invest* **32,** 704–716.

Shimoda LA, Sylvester JT & Sham JS (1998). Inhibition of voltage-gated K+ current in rat intrapulmonary arterial myocytes by endothelin-1. *Am J Physiol* **274,** L842-853.

Shovlin CL (2014). Iron deficiency, ischaemic strokes, and right-to-left shunts: From pulmonary arteriovenous malformations to patent foramen ovale? *Intractable Rare Dis Res* **3,** 60–64.

Siebenmann C, Keiser S, Robach P & Lundby C (2017). CORP: The assessment of total hemoglobin mass by carbon monoxide rebreathing. *Journal of Applied Physiology* **123,** 645–654.

Silva M, Grillot D, Benito A, Richard C, Nuñez G & Fernández-Luna JL (1996). Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* **88,** 1576–1582.

Skattebo Ø & Hallén J (2022). Individual variations in pre-altitude hemoglobin mass influence hemoglobin mass responses to repeated altitude sojourns. *Scand J Med Sci Sports* **32,** 1493–1501.

Slaats J, ten Oever J, van de Veerdonk FL & Netea MG (2016). IL-1β/IL-6/CRP and IL-18/ferritin: Distinct Inflammatory Programs in Infections. *PLoS Pathog* **12,** e1005973.

Sommer N, Dietrich A, Schermuly RT, Ghofrani HA, Gudermann T, Schulz R, Seeger W, Grimminger F & Weissmann N (2008). Regulation of hypoxic pulmonary vasoconstriction: basic mechanisms. *Eur Respir J* **32,** 1639–1651.

Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, Trembath RC, Jennings S, Barker L, Nicklin P, Walker C, Budd DC, Pepke-Zaba J & Morrell NW (2010). Elevated Levels of Inflammatory Cytokines Predict Survival in Idiopathic and Familial Pulmonary Arterial Hypertension. *Circulation* **122,** 920–927.

Spitzer JA (1999). Gender differences in some host defense mechanisms. *Lupus* **8,** 380–383.

Strunk BL, Cheitlin MD, Stulbarg MS & Schiller NB (1987). Right-to-left interatrial shunting through a patent foramen ovale despite normal intracardiac pressures. *Am J Cardiol* **60,** 413–415.

Subudhi AW et al. (2014). AltitudeOmics: The Integrative Physiology of Human Acclimatization to Hypobaric Hypoxia and Its Retention upon Reascent. *PLOS ONE* **9,** e92191.

Sureda A, Batle JM, Capó X, Martorell M, Córdova A, Tur JA & Pons A (2014). Scuba diving induces nitric oxide synthesis and the expression of inflammatory and regulatory genes of the immune response in neutrophils. *Physiol Genomics* **46,** 647–654.

Swann JW, Koneva LA, Regan-Komito D, Sansom SN, Powrie F & Griseri T (2020). IL-33 promotes anemia during chronic inflammation by inhibiting differentiation of erythroid progenitors. *Journal of Experimental Medicine* **217,** e20200164.

Swenson ER (2013). Hypoxic pulmonary vasoconstriction. *High Alt Med Biol* **14,** 101–110.

Sylvester JT, Shimoda LA, Aaronson PI & Ward JPT (2012). Hypoxic pulmonary vasoconstriction. *Physiol Rev* **92,** 367–520.

Talbot NP, Balanos GM, Dorrington KL & Robbins PA (2005). Two temporal components within the human pulmonary vascular response to approximately 2 h of isocapnic hypoxia. *J Appl Physiol (1985)* **98,** 1125–1139.

Tan C, Rubenson D, Srivastava A, Mohan R, Smith MR, Billick K, Bardarian S & Thomas Heywood J (2017). Left ventricular outflow tract velocity time integral outperforms ejection fraction and Doppler-derived cardiac output for predicting outcomes in a select advanced heart failure cohort. *Cardiovasc Ultrasound* **15,** 18.

Tarumi T, Sawada K, Sato N, Kobayashi S, Takano H, Yasukouchi T, Takashashi T, Sekiguchi S & Koike T (1995). Interferon-alpha-induced apoptosis in human erythroid progenitors. *Exp Hematol* **23,** 1310–1318.

Thom SR, Milovanova TN, Bogush M, Yang M, Bhopale VM, Pollock NW, Ljubkovic M, Denoble P, Madden D, Lozo M & Dujic Z (2013). Bubbles, microparticles, and neutrophil activation: changes with exercise level and breathing gas during open-water SCUBA diving. *J Appl Physiol (1985)* **114,** 1396–1405.

Timmons BW, Hamadeh MJ, Devries MC & Tarnopolsky MA (2005). Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. *Journal of Applied Physiology* **99,** 979–985.

Topiwala KK, Patel SD, Pervez M, Shovlin CL & Alberts MJ (2021). Ischemic Stroke in Patients With Pulmonary Arteriovenous Fistulas. *Stroke* **52,** e311–e315.

Torcia MG, Nencioni L, Clemente AM, Civitelli L, Celestino I, Limongi D, Fadigati G, Perissi E, Cozzolino F, Garaci E & Palamara AT (2012). Sex differences in the response to viral infections: TLR8 and TLR9 ligand stimulation induce higher IL10 production in males. *PLoS One* **7,** e39853.

Tóthová Z, Tomc J, Debeljak N & Solár P (2021). STAT5 as a Key Protein of Erythropoietin Signalization. *Int J Mol Sci* **22,** 7109.

Tsai BM, Wang M, Pitcher JM, Meldrum KK & Meldrum DR (2004). Hypoxic pulmonary vasoconstriction and pulmonary artery tissue cytokine expression are mediated by protein kinase C. *Am J Physiol Lung Cell Mol Physiol* **287,** L1215-1219.

Valic Z, Duplancić D, Baković D, Ivancev V, Eterović D, Wisløff U, Brubakk AO & Dujić Z (2005). Diving-induced venous gas emboli do not increase pulmonary artery pressure. *Int J Sports Med* **26,** 626–631.

Vanderpool RR & Naeije R (2018). Hematocrit-corrected Pulmonary Vascular Resistance. *Am J Respir Crit Care Med* **198,** 305–309.

Vannucchi AM, Grossi A, Rafanelli D, Statello M, Cinotti S & Rossi-Ferrini P (1994). Inhibition of erythropoietin production in vitro by human interferon gamma. *Br J Haematol* **87,** 18–23.

Vik A, Jenssen BM, Eftedal O & Brubakk AO (1993). Relationship between venous bubbles and hemodynamic responses after decompression in pigs. *Undersea Hyperb Med* **20,** 233–248.

Wang CQ, Udupa KB & Lipschitz DA (1995). Interferon-γ exerts its negative regulatory effect primarily on the earliest stages of murine erythroid progenitor cell development. *Journal of Cellular Physiology* **162,** 134–138.

Wang CQ, Udupa KB & Lipschitz DA (1996). Evidence suggesting a stimulatory role for interleukin-10 in erythropoiesis in vitro. *J Cell Physiol* **166,** 305–310.

Wang J, Ji Y, Zhou L, Xiang Y, Heinonen I & Zhang P (2019*a*). A New Method to Improve Running Economy and Maximal Aerobic Power in Athletes: Endurance Training With Periodic Carbon Monoxide Inhalation. *Front Physiol* **10,** 701.

Wang L, Liu J, Wang W, Qi X, Wang Y, Tian B, Dai H, Wang J, Ning W, Yang T & Wang C (2019*b*). Targeting IL-17 attenuates hypoxia-induced pulmonary hypertension through downregulation of β-catenin. *Thorax* **74,** 564–578.

Weir EK & Archer SL (1995). The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB J* **9,** 183–189.

Weir EK, López-Barneo J, Buckler KJ & Archer SL (2005). Acute oxygen-sensing mechanisms. *N Engl J Med* **353,** 2042–2055.

Weiss G, Ganz T & Goodnough LT (2019). Anemia of inflammation. *Blood* **133,** 40–50.

Wessling-Resnick M (2010). Iron homeostasis and the inflammatory response. *Annu Rev Nutr* **30,** 105–122.

West JB (2011). History of respiratory gas exchange. *Compr Physiol* **1,** 1509–1523.

West JB (2015). *Essays on the history of respiratory physiology*. Springer, New York ; Heidelberg.

West JB, Schoene RB, Luks AM & Milledge JS (2013). *High Altitude Medicine and Physiology*, 5th edn. Available at: https://www.taylorfrancis.com/pdfviewer/ [Accessed January 19, 2023].

Wojan F, Stray-Gundersen S, Nagel MJ & Lalande S (2021). Short exposure to intermittent hypoxia increases erythropoietin levels in healthy individuals. *J Appl Physiol (1985)* **130,** 1955–1960.

Wrighting DM & Andrews NC (2006). Interleukin-6 induces hepcidin expression through STAT3. *Blood* **108,** 3204–3209.

Wu Z, Giudice V, Chen J, Sun W, Lin Z, Keyvanfar K, Talasani N, Kajigaya S, Feng X & Young NS (2019). Interleukin-18 plays a dispensable role in murine and likely also human bone marrow failure. *Exp Hematol* **69,** 54-64.e2.

Xiao W, Koizumi K, Nishio M, Endo T, Osawa M, Fujimoto K, Sato I, Sakai T, Koike T & Sawada K ichi (2002). Tumor necrosis factor-alpha inhibits generation of glycophorin A+ cells by CD34+ cells. *Exp Hematol* **30,** 1238–1247.

Xu D & Huang S-K (2020). IL-33: a key player in the development of iron-recycling red pulp macrophages. *Cell Mol Immunol* **17,** 1218–1219.

Yock PG & Popp RL (1984). Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. *Circulation* **70,** 657–662.

Žarak M, Perović A, Njire Bratičević M, Šupraha Goreta S & Dumić J (2021). Adaptive response triggered by the repeated SCUBA diving is reflected in cardiovascular, muscular, and immune biomarkers. *Physiol Rep* **9,** e14691.

Zavorsky GS, Tesler J, Rucker J, Fedorko L, Duffin J & Fisher JA (2014). Rates of carbon monoxide elimination in males and females. *Physiol Rep* **2,** e12237.