ASSOCIATION OF PATENT FORAMEN OVALE, INFLAMMATORY CYTOKINES, AND WHITE BLOOD CELLS WITH HEMOGLOBIN MASS IN MEN

by

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A patent foramen ovale (PFO) is a source of intracardiac shunt, and preliminary data suggests that those with a PFO have higher concentrations of inflammatory cytokines. Cytokines and white blood cells (WBCs) negatively impact red blood cell (RBC) regulation, but no studies have examined the effect of the PFO on this relationship. The purpose of this study was to investigate the relationships between the presence of a PFO, inflammatory cytokine concentrations, and WBCs on RBC mass. We hypothesized that those with a PFO would have both a higher WBC count and cytokine concentrations, negatively impacting hemoglobin (Hb) mass. Twenty healthy, male participants completed the study (10 with and 10 without a PFO). Participants underwent a comprehensive ultrasound screening with saline contrast echocardiography to determine the presence or absence of a PFO. Hb mass was measured twice on the same day via the 10-minute CO-rebreathe method, and venous blood samples were drawn for measurements of WBC counts and inflammatory cytokines. WBC counts were analyzed by QUEST diagnostics via flow cytometry. Cytokine analysis was done using the BioLegend 13-plex human inflammation panel and analyzed via flow cytometry. No differences were found for absolute (g) ($p = 0.1096$) and relative (g/kg) ($p = 0.1382$) Hb mass between PFO+ and PFO- participants. No differences were found for WBC count ($p = 0.8680$) between PFO+ and PFO-

participants and for each of the respective immune cells; neutrophils ($p = 0.5418$), lymphocytes ($p = 0.6721$), monocytes ($p = 0.5388$), eosinophils ($p = 0.5603$), basophils ($p = 0.2539$). There was no differences in cytokine concentrations between PFO+ and PFO- participants. There was no significant relationship between WBC count and absolute or relative Hb mass. There was a relationship between IFN-α2 and the whole group data for absolute Hb mass ($p = 0.0390$, $R^2 = 0.2158$). There was a relationship between MCP-1 levels and absolute Hb mass in PFO+ participants, but not in PFO- participants ($p = 0.0258$, $R^2 = 0.4823$). Similarly, there was a relationship between IL-23 levels and absolute Hb mass in PFO+ participants, but not in PFO- participants ($p = 0.0497$, $R^2 = 0.5003$). Conversely, there was a relationship between IL-10 levels and absolute Hb mass in PFO- participants, but not in PFO+ participants ($p = 0.0392$, $R^2 = 0.5350$). Although there were no PFO differences in Hb mass, WBC counts or cytokine concentrations, the presence of a PFO may alter the relationship between Hb mass and the cytokines MCP-1, IL-23 and IL-10. In addition, we found a significant relationship with IFN-α2 and absolute Hb mass irrespective of the presence or absence of a PFO, indicating a potential role for IFN-α2 on Hb mass regulation.

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Abbreviations

AI: Anemia of Inflammation

CO: Carbon Monoxide

CO_2: Carbon Dioxide

CV: Coefficient of Variation

EPO: Erythropoietin

PFO: Patent Foramen Ovale

PFO+: With a Patent Foramen Ovale

PFO-: Without a Patent Foramen Ovale

EDTA: Ethylenediaminetetraacetic Acid

Hb: Hemoglobin

HbCO: Carboxyhemoglobin

HIF: Hypoxia Inducible Factor

HSC: Hematopoietic Stem Cells

IFN: Interferon

IL: Interleukin

IV: Intravenous

MCP: Monocyte Chemoattractant Protein

O_2: Oxygen

RBC: Red Blood Cell

TNF: Tumor Necrosis Factor

WBC: White Blood Cell

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Introduction

Blood is a fluid that moves through the vessels of our circulatory system. The main components of blood are plasma, red blood cells (RBC), white blood cells (WBC) and platelets. Plasma serves as a transport medium for various cells and substances to be distributed throughout the body. RBCs are required for the transport of oxygen (O_2), carbon dioxide (CO_2) and other molecules such as carbon monoxide (CO), and does this through the protein hemoglobin (Hb). Hb mass is the absolute mass of circulating Hb in the body and gives us a way to measure RBC mass. Interestingly, it is known that Hb has a high affinity for CO and can replace O_2 at sea level barometric pressure. WBCs play an important role in our immune system and ability to mount an immune response. Some types of WBCs include neutrophils, monocytes, eosinophils, lymphocytes and basophils. These cells are able to recognize and engulf pathogens and release proteins called cytokines that regulate the immune system and its inflammatory response. All of these WBCs are able to produce numerous types of pro and anti-inflammatory cytokines depending on the physiological context. Pro-inflammatory cytokines activate the immune system while anti-inflammatory cytokines suppress the immune response and help keep the actions of pro-inflammatory cytokines in check. The cytokines released by WBCs travel in our plasma to be delivered throughout the body and act to stimulate or inhibit the activation, proliferation and differentiation of various immune cells. Cytokines are markers of inflammation in the body, so higher levels of cytokines in the blood indicate higher levels of inflammation. Inflammation can impact other aspects outside of the immediate immune response. Notably, inflammation can alter RBC regulation through the release of various cytokines. The main mechanisms through which cytokines can alter RBC mass are altering iron availability, inhibiting or stimulating erythropoietin (EPO), and acting on erythroid progenitor

cells or RBCs directly (Johnson et al., 1990; Fandrey & Jelkmann, 1991; Faquin et al., 1992; Lai et al., 1995; Tarumi et al., 1995; Dai et al., 1998; La Ferla et al., 2002; Fleming, 2008; Libregts et al., 2011).

While in utero, the fetus has a foramen ovale, which is a small hole between the upper chambers of the heart that allows blood to bypass the lungs while the fetus is still receiving oxygen from the mother. The foramen ovale typically closes after birth, but in ~25-40% of the population, this tunnel remains open, or patent, and we term it patent foramen ovale (PFO) (Marriott, et. al., 2013). A PFO is associated with various pathologies, such as increased risk of stroke and migraine with aura, and our lab has shown that those with a PFO respond differently to various environmental stressors like heat and altitude (Davis et al., 2017; Davis et al., 2015; Davis et al., 2019; Lovering et al., 2016; Lovering et al., 2022). In addition, preliminary data from our lab has shown that individuals with a PFO (PFO+) have higher levels of circulating cytokines, suggesting increased inflammation in the body. While is it known that WBCs release cytokines and are involved with inflammation, no studies have yet looked at the differences in WBC count in subjects with and without a PFO. In addition, no current studies have looked at the relationship between the PFO, WBC count and cytokine concentrations with respect to Hb mass.

The purpose of my study was to investigate the associations between WBCs, cytokines, and the PFO on Hb mass in men. We hypothesized that those with a PFO would have both a higher WBC count and cytokine concentrations, negatively impacting Hb mass.

Literature Review

Cardiopulmonary Physiology

The cardiovascular system is composed of pulmonary and systemic circulations. Pulmonary circulation directs blood to the lungs where it participates in gas exchange, while systemic circulation directs blood throughout the body to supply oxygen and nutrients to various tissues and organs. In normal circulation without shunts, venous blood enters the right atrium via the superior and inferior vena cava and coronary sinus. From there, it is pumped to the right ventricle and to the lungs via the pulmonary arteries. At the lungs, gas exchange occurs across the alveoli into the capillaries and vice versa. During pulmonary gas exchange, oxygen diffuses into the blood to be transported throughout the body. Simultaneously, as oxygen diffuses into the blood, carbon dioxide diffuses out of the blood and into the alveoli to be exhaled from the body. After passing through the lungs, blood returns to the left atrium via the pulmonary veins. From there, oxygenated blood enters the left ventricle, is pumped into the aorta, and is delivered throughout the rest of the body. However, humans have various shunts, so the small amount of blood flow traveling through those shunts will not come in contact with the lung alveoli, and instead will be directed straight into the pulmonary veins and left atrium. One shunt pathway of particular interest to our lab is the PFO, which will be discussed in more detail below.

The main components of blood are plasma, erythrocytes (red blood cells; RBC), leukocytes (white blood cells; WBC) and platelets. Plasma makes up roughly 55% of blood and is composed of mostly water with various proteins, ions and nutrients. Erythrocytes make up approximately 45% of blood and are primarily responsible for O_2 and CO_2 transport via the protein Hb, which will be discussed in more detail below. Platelets and WBCs make up less than 1% of blood. Platelets are cell fragments that aid in blood clotting. WBCs play an important

role in our immune system by recognizing and engulfing pathogens and releasing proteins called cytokines that help regulate the intensity and duration of our immune response. The focus of this thesis is on the associations of the PFO, cytokines, WBCs, and RBCs, so I will begin with discussing erythrocyte and immune system functions, followed by what is known about the interactions between the erythropoietic and immune systems. Lastly, the PFO will be discussed.

Erythrocyte function

RBCs contain the ferric protein Hb, which is responsible for carrying O_2, CO_2, and other gaseous molecules such as CO throughout the body. Hb is comprised of two alpha and two beta subunits surrounding an iron-containing heme group. Hb can bind up to four units of O_2 or other gaseous molecules, and the binding affinity increases as more gas molecules are bound to the heme group, which is referred to as cooperativity (Eaton et al., 1999).

O_2 is transported in the blood through two mechanisms. It binds to Hb and also directly dissolves in the blood. Roughly 98.5% of O_2 binds to Hb and the remainder is dissolved in the blood (Molnar & Gair, 2015). Therefore, the amount of Hb is functionally significant for regulating O_2 transport.

CO_2 is transported in the blood through three mechanisms. It can bind to Hb, dissolve directly in the blood, and be carried as bicarbonate. The bicarbonate buffer system accounts for 85% of CO_2 transport (Molnar & Gair, 2015). In this system, CO_2 diffuses into RBCs and is converted to carbonic acid by carbonic anhydrase. Carbonic acid then quickly dissociates into bicarbonate and hydrogen ions and is able to buffer pH changes in the blood. Once bicarbonate reaches the lungs, it is converted to CO_2 and water, and exhaled from the body. Only about 10% of CO_2 is transported bound to Hb, so Hb has a more significant in O_2 transport.

In addition to O_2 and CO_2, Hb can bind CO. However, CO binds more tightly to Hb than oxygen and can replace O_2 when present in sufficient amounts. When CO is present, it preferentially binds to Hb, preventing the binding and transport of O_2 (Molnar & Gair, 2015). In the short term, elevated levels of CO in the blood result in CO poisoning leading to symptoms such as headaches, confusion and nausea. In the long term, elevated levels of CO can cause brain damage and death.

The carbon monoxide (CO) rebreathe method is the gold standard for measuring Hb mass, and has been used extensively by many research labs (Thomsen et al., 1991; Burge & Skinner, 1995; Garvican et al., 2010; Siebenmann et al., 2017). Hb mass is defined as the absolute mass of circulating Hb. It offers insight about the amount of functional RBCs able to bind and transport O_2 in the body and gives us a way to measure RBC mass. The optimized CO rebreathing method uses the increased binding affinity of CO to calculate the total number of circulating Hb molecules based on the amount of CO absorbed and change in CO bound to Hb, also known as carboxyhemoglobin (HbCO) (Burge & Skinner, 1995; Garvican et al., 2010; Siebenmann et al., 2017).

Erythrocyte formation and regulation

Hematopoiesis is the process of producing cellular blood components. Erythropoiesis is a specific type of hematopoiesis that gives rise to RBCs. During this process, hematopoietic stem cells (HSC) differentiate into erythroid progenitors, and eventually mature into RBCs. Erythroid progenitors are cells that commit to only becoming RBCs. The process of erythropoiesis is responsible for maintaining RBC levels within normal physiological range. The physiological range for a healthy, adult male is 4.7 to 6.1 million RBCs per microliter of blood. RBC homeostasis is highly regulated to ensure adequate oxygenation throughout the body and is

important for maintaining a healthy blood pressure and blood viscosity. To do this, RBCs are constantly being produced and recycled. The lifespan of an RBC is approximately 120 days; therefore, millions of RBCs must constantly be made through the process of erythropoiesis to maintain normal levels. Macrophages from the mononuclear phagocytic system (MPS) phagocytose old or damaged RBCs as they pass through sinusoids of the spleen and liver (Arias & Arias, 2017).

Erythropoiesis is primarily regulated by the cytokine erythropoietin (EPO) and is supported by secondary cytokines, hormones and transcriptional factors (Jelkmann, 2011). EPO is required for immature cells to commit to becoming mature RBCs. To do this, EPO binds to receptors on erythroid progenitor cells and triggers differentiation and maturation into functional RBCs (Jelkmann, 2011). EPO is primarily produced in the kidneys and to a lesser degree in the liver. EPO is expressed at low levels at sea level to maintain RBC mass during normal RBC turnover.

In response to low blood oxygen content, the kidneys and liver will increase EPO production. Acutely, when there is a reduction in oxygen delivery to the kidneys and liver, hypoxia inducible factor (HIF) pathways promote an increase in EPO production such that the HIF transcriptional factor is required for the transcription of EPO. HIF pathways also enhance iron uptake and utilization, regulate iron metabolism, and promote expression of erythroid progenitors, which are all important responses to increase RBC mass (Haase, 2013). Iron is an important factor in regulating RBC production, and since Hb is a ferric protein, iron is crucial in the formation of functional RBCs. If the hypoxic stimulus is maintained chronically, EPO will stimulate the production of more RBCs. This increase in RBC production improves O_2 carrying capacity and enhances O_2 delivery. Many hypoxia studies have been conducted by taking

participants to higher altitudes where blood oxygen saturation decreases. These studies consistently show a dose and time dependent increase in EPO concentration. In a study conducted by Faura et al (1969), subjects were taken to high altitude for 7 days. Upon exposure to altitude, EPO concentration began to increase from baseline after a lag period of 6 hours, followed by a continuous marked increase in EPO concentration over the next 24 hours, before returning to basal levels. This trend is consistent with the data found in animals models, where EPO concentration peaked between 8-18 hours and returned back to baseline within 48-72 hours following hypoxic stimulus (Erslev, 1957; Stohlman, 1959; Prentice & Mirand, 1961). Acutely, 120 minutes of continuous hypoxia or eight 4-minute cycles of intermittent hypoxia were sufficient to induce increases in EPO (Wojan et al. 2021). Despite similar trends in EPO increase following hypoxia exposure, it is important to consider the variability in EPO response between individuals based on factors such as iron stores, nutrition and pathophysiological conditions (Grover et al., 1998). In addition, while it has been shown that EPO increases in both men and women at higher altitudes, it is clear that the hematopoietic response to hypoxia at altitude in men has been studied more in depth (Faura et al, 1969; Abbrecht & Littell, 1972; Reeves et al., 2001; Ge et al., 2002; Wojan et al., 2021).

Immune system

The human immune system can be divided into the innate and adaptive immune systems. The innate immune system is the immediate, non-specific response to harmful stimuli and involves the action of numerous circulating cells and proteins. It consists of physical barriers such as the skin and mucous membranes, as well as numerous immune cells, including phagocytes (Sherwood, 2015). The adaptive immune system is specific and has the capacity for immunological memory. The adaptive immune system uses both antibody mediated immunity

with B-cells and cell mediated immunity with T cells to mount a tailored response for a specific threat (Sherwood, 2015).

Immune cells, also known as WBCs, are produced through leukopoiesis, a specific type of hematopoiesis. In addition to the erythroid cell line, HSCs give rise to the myeloid and lymphoid cell lines. The myeloid cells form granulocytes, phagocytes, platelets and erythrocytes (Owen et al., 2013). Types of granulocytes include neutrophils, basophils, and eosinophils. These cells are classified together because they all have cytoplasmic granules containing effector molecules that aid in the immune response once released (Geering et al., 2013). Types of phagocytes include macrophages, monocytes and dendritic cells. These cells are important for phagocytosis, which is the process of engulfing and digesting foreign particles or old cells. Phagocytes also assist in presenting antigens to initiate the adaptive immune response (Owen et al., 2013). The lymphoid cell line forms both B and T lymphocytes. B cells secrete antibodies, which are protective proteins of the immune system that bind to and neutralize pathogens and toxins (Mauri & Bosma, 2012). Antibodies are highly specific to the proteins expressed on the surface of pathogens, allowing antibodies to recognize and eliminate cells with the corresponding protein. T cells have multiple subtypes, but all play an important role in recognizing antigens and producing cytokines that activate other immune cells. Some types of T cells, called cytotoxic T cells, directly eliminate pathogens or infected cells (Sherwood, 2015).

As noted above, one of the main functions of WBCs is to release cytokines. Cytokines can also be produced by endothelial and epithelial cells, adipocytes, and connective tissue (Arango Duque & Descoteaux, 2014). Cytokines are proteins that act as chemical messengers to regulate the immune system and its inflammatory response. They are responsible for promoting and regulating the activity, differentiation, proliferation and production of other cells and

cytokines involved in the immune system (Ferreira, 2018). When cytokines are released, they travel in our plasma to be delivered throughout the body (Clark et al., 2018). Cytokines can also act on their target cells in an autocrine or paracrine fashion. Once released, cytokines bind to specific receptors on the plasma membrane of target cells. These cytokines initiate a signaling cascade, leading to alterations in protein activity and gene expression. As a result, cytokines stimulate or inhibit the activation, proliferation and differentiation of various immune cells. They also regulate the secretion of other cytokines and antibodies and can induce cell death (Owen et al., 2013). Pro-inflammatory cytokines promote inflammation by increasing capillary permeability and leukocyte migration at the site of damage as well as initiate the systemic fever response. Conversely, anti-inflammatory cytokines suppress the immune response and help keep the actions of pro-inflammatory cytokines in check.

Cytokines can be divided into six different families based on common structure and function. These families include: the Interleukin 1 family (IL-1), the hematopoietin family (Class I cytokine), the Interferon family (Class II cytokine), the Tumor Necrosis Factor family (TNF), the Interleukin 17 family (IL-17), and the Chemokine family (Owen et al., 2013). The following cytokines will be analyzed in this study and discussed in more depth below: 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.

Specific to this study, IL-1β, IL-18 and IL- 33 are all members of the Interleukin 1 family. Cytokines in this family are released following the recognition of a foreign invader by cells involved in the innate immune system and carry out pro-inflammatory effects in the body. Some of these effects include increased capillary permeability at the site of cytokine secretion,

amplification of leukocyte emigration, signaling for other cytokine and chemokine production and activation of cells involved in the adaptive immune system (Owen et al., 2013).

IL-6, IL-23 and IL- 12p70 are all members of the hematopoietin family. While these cytokines carry out various functions in the immune system, they are grouped together based on similarities in their structure and signaling pathways. IL-6 has both pro and anti-inflammatory effects while IL-23 and IL-12p70 are both pro-inflammatory cytokines. As mentioned previously, EPO is also a cytokine and falls within the hematopoietin family. It acts as an anti-inflammatory cytokine and is required for the proliferation, differentiation and survival of erythroid progenitor cells to become mature RBCs. Given that EPO promotes increased differentiation into erythroid progenitors, fewer cells will commit to becoming WBCs. Some of the anti-inflammatory effects of EPO can be explained by this decrease in WBC population and therefore decreased release of cytokines (Paulson et al., 2020).

As mentioned above, IFN- $\alpha 2$ and γ are both classified in the interferon family and function by interfering with viral replication. IFN-$\alpha 2$ and IFN-γ both act in a pro-inflammatory and anti-inflammatory fashion. IFN-$\alpha 2$ is produced by infected immune cells while IFN- γ responds to the secretion of cytokines from other immune cells such as activated antigen presenting cells (Schroder, 2004). IFN-$\alpha 2$ carries out anti-viral effects through autocrine and paracrine signaling in which viral replication of infected cells is suppressed and the interferon response of nearby cells is enhanced to protect against potential viral infection (Khan, 2019; Michael Lavigne et al., 2021). IFN-γ is known as the primary macrophage activator and aids in pathogen recognition, antigen processing and presentation, WBC attraction, cell growth inhibition and programmed cell death (Schroder, 2004). IL-10 is also grouped in the Interferon family given the structural similarities with IFN- γ and ability to bind to the same class of

receptors. IL-10 carries out anti-inflammatory effects and is most important in immune suppression (Commins et al., 2008).

TNF-α is a pro-inflammatory cytokine apart of the Tumor Necrosis family and is produced by various immune cells such as macrophages, monocytes and various T cell subtypes (Khan, 2019). TNF-α enhances immune cell aggregation to blood vessel walls, induces chemokine production to aid in the destruction of infected cells and promotes programmed cell death (Idriss & Naismith 2000; Khan, 2019).

IL-17A is a part of the Interleukin 17 family. It acts as a pro-inflammatory cytokine that mounts an immune response. In particular, it coordinates the release of other pro-inflammatory cytokines and enhances neutrophil recruitment (Owen et al., 2013).

MCP-1 is a pro-inflammatory cytokine that is part of the chemokine family. It is produced by epithelial and endothelial cells, fibroblasts and smooth muscle, as well as a variety of cells in response to oxidative stress, stimulation by other cytokines, and growth factors (Deshmane et al., 2009). As mentioned above, chemokines are responsible for migration of various immune cells during an immune response. Out of the antigen presenting cells, MCP-1 has the greatest effect on monocytes and macrophages. The primary functions of MCP-1 are to selectively recruit and regulate monocyte, neutrophil and lymphocyte migration and infiltration (Deshmane et al., 2009). IL-8 is also a part of the chemokine family and plays a major role in neutrophil regulation and function (Mukaida et al., 1998).

Immune system regulation of erythrocytes

Inflammation has many widespread effects beyond the immediate immune response. For example, inflammation causes an increase in macrophage activity, resulting in macrophages recycling more RBCs than normal and decreasing the number of circulating RBCs (Klei *et al.*,

2017). Overactivity of the immune system resulting in a clinical reduction of RBC mass is known as anemia of inflammation (AI). AI is associated with disturbances in erythropoiesis and causes a reduction in RBC survival (Weiss & Goodnough, 2005).

Immune cells and the cytokines they release can negatively affect erythropoiesis and RBC mass through multiple pathways. The main ways inflammation can impact RBC mass is through inhibition of EPO production, limiting iron availability, and disrupting erythroid precursor and/or RBC survival and proliferation (Nemeth et al., 2004; Lee et al., 2005; Fleming, 2008). The role of IL-8, IL-18, IL-23 and MCP-1 on erythropoiesis is not completely understood. However, below I will summarize research that shows how some of the cytokines studied in this thesis are known to alter RBC homeostasis.

IL-1β has been shown to cause a dose-dependent inhibition of EPO (Fandrey & Jelkmann, 1991; Faquin et al., 1992; La Ferla et al., 2002). In addition to its ability to inhibit EPO production, IL-1β has also been shown to inhibit the proliferation of erythroid precursors in mouse spleen and bone marrow cells (Schooley et al., 1987). Similarly, TNF-α has also been shown to inhibit EPO (Johnson et al., 1990; Fandrey & Jelkmann, 1991; Faquin et al., 1992; La Ferla et al., 2002).

IL-6 is known to increase transcription of the protein hepcidin which is responsible for regulating iron homeostasis. Hepcidin regulates iron levels by inhibiting iron absorption in the small intestine and preventing iron release from macrophages (Nemeth et al., 2004; Lee et al., 2005). As a result, an increase in IL-6 leads to a decrease in circulating iron available for RBC synthesis (Fleming, 2008).

IL-10 is an anti-inflammatory cytokine, and acts to suppress the immune system through inhibition of other cytokines and immune cells. In line with its anti-inflammatory properties,

there is some support of a dose-dependent IL-10 inhibition of erythroid progenitors in human blood cells (Oehler et al., 1999). However, the data regarding the effect of IL-10 on erythropoiesis is inconsistent. When added to mouse cell cultures, there was an increase in erythroid progenitor cell colonies, and when combined with EPO, IL-10 had a stimulatory role on erythropoiesis (Wang et al., 1996). Similarly, Rennick et al (1994) showed that IL-10 regulates stages of hematopoietic development by increasing the formation of erythroid progenitor cell colonies when used in combination with EPO and other cytokines.

The role of IL-12 is greatly dependent on the environment and its interactions with other cytokines. One study suggests that in vitro, IL-12 can stimulate erythropoiesis in the presence of EPO by increasing the number of early-stage erythroid precursors (Dybedal et al., 1995). However, when conducted in vivo, IL-12 was shown to suppress erythropoiesis through increasing the expression of IFN-γ (Eng et al., 1995). IFN-γ has been shown to have inhibitory effects on RBC precursors when present on its own and stimulatory effects on these precursors when combined with other cytokines such as IL-1 and TNF-α (Vannucchi et al., 1994). IFN- γ can also induce cell death of erythroid progenitors and reduce both the lifespan of RBCs and the formation of new ones (Dai et al., 1998; Libregts et al., 2011). IFN-α2 has also been shown to impact erythroid progenitors by inhibiting their proliferation and inducing cell death (Lai et al., 1995; Tarumi et al., 1995). Similar to IL-12, the microenvironment determines which effects IL-17 has on different erythroid progenitors. It has been shown to promote the earliest erythroid precursors while inhibiting the rare bone marrow precursors (Krstic et al., 2012; Mojsilović et al., 2015). However, it can promote these rare bone marrow precursors in the spleen, while inhibiting them in the bone marrow (Mojsilović et al., 2015).

IL-33 has been shown to suppress erythropoiesis by acting directly on erythroid progenitors in a mouse model (Swann et al., 2020). In addition, IL-33 plays an important role in the production of splenic red pulp macrophages that are required for iron recycling in erythrocyte homeostasis (Lu et al., 2020; Xu & Huang, 2020). Therefore, elevated IL-33 levels may speed up the process of recycling RBCs, impacting RBC mass.

Fetal circulation & Patent Foramen Ovale

In utero, the structure of fetal circulation is different than an adult and allows gas exchange to occur at the level of the placenta rather than at the lungs. To allow oxygenated blood to bypass the lungs in utero, the fetal circulation has three distinct shunt pathways: the ductus venosus, the ductus arteriosus, and the foramen ovale. These fetal shunts divert blood in response to pressure gradients and are responsible for directing the gas exchange from the mother's blood to the fetal blood. Placental circulation is crucial for development of the fetal cardiopulmonary system and provides a low-resistance pathway between the mother and the fetus for gas exchange and nutrient delivery (Finnemore & Groves, 2015).

Given that the fetus cannot breathe independently in the womb, the environment is extremely hypoxic and requires oxygen from the mother. This fetal hypoxemia promotes vasoconstriction within the pulmonary circulation, increasing right atrial pressure compared to that of left atrial pressure and favoring the passage of blood through the foramen ovale into the left atrium. Oxygenated and nutrient-rich blood from the placenta passes to the fetus via the umbilical vein, and about half of this blood bypasses the liver via the ductus venosus and enters through the inferior vena cava into the right atrium (Gilroy et al., 2017). The increased pressure in the right atria favors passage of blood through the foramen ovale into the left atria, bypassing pulmonary circulation. Blood that did not pass through the foramen ovale continues through the

right ventricle and into the pulmonary trunk where the ductus arteriosus diverts it into the aorta, ensuring oxygenated blood goes directly into systemic circulation (Huff et al., 2021). Of particular interest to this thesis is the foramen ovale.

When the child takes their first breath of ambient air outside the womb, changes in the pressure gradients across the right and left atria favor closure of the foramen ovale structure in most people. As discussed before, fetal hypoxemia occurs while in the womb resulting in greater right atrial pressure compare to that of left atrial pressure. After breathing ambient air, the child can breathe independently, resolving fetal hypoxemia. This causes pulmonary vasoconstriction and right atrial pressure to decrease, closing the shunt. However, in about 25-40% of the population, the foramen ovale does not close for unknown reasons and is termed a PFO (Marriott, et. al., 2013).

A PFO is a tunnel between the right and left atria of the heart and is a source of intracardiac shunt. Depending on PFO size and the pressure gradient across the atria, 5-10% of cardiac output in the healthy population can be shunted through a PFO (Davis et al., 2015). With a PFO, some blood can flow straight from the right atrium to the left atrium, skipping the lungs and immediately entering the systemic circulation.

Many studies by our lab have been conducted to investigate the associations of a PFO on the cardiopulmonary, respiratory and thermoregulatory systems (Davis et al., 2015, 2017, 2019; Lovering et al., 2012, 2015, 2016, 2022). It has been shown that those with a PFO have greater gas exchange inefficiency given that some blood bypasses pulmonary circulation at the lungs (Lovering et al., 2016). As a result, this shunted blood is not able to get oxygenated and leads to a decrease in oxygen saturation of circulating blood. In addition, respiratory cooling occurs at the lungs and allows the dissipation of heat (Lovering et al., 2015). Those with a PFO have a

higher core body temperature of about ~0.4 ℃ because some of their blood is not cooled at the lungs (Lovering et al., 2016). PFOs have also been associated with many pathophysiological conditions such as migraine, cryptogenic stroke and transient ischemic attack (Lovering et al., 2022). In addition, preliminary data from our laboratory suggests that those subjects with a PFO have higher baseline inflammation; however, the catalyst for this observation has not been well-studied.

Methods

Participants

All procedures detailed below were approved by the Institutional Review Board at the University of Oregon. Prior to enrollment, participants underwent an informed consent process. All experimental procedures were conducted in accordance of the Declaration of Helsinki. This study is part of a larger project with various aims examining Hb mass, exercise, and breathing responses at sea level and altitude. For this study, twenty male participants were screened and enrolled, 10 with a PFO and 10 without a PFO (see below for details about PFO screening). Characteristics of the participants who completed the study are – age: 26.2 ± 4.8 years; height: 180.7 ± 4.6 cm; and weight: 77.4 ± 9.5 kg. All participants were healthy, active and free from underlying cardiopulmonary health concerns.

Data collection procedure

For this study, participants completed two data collection days. On the first, participants were screened for the presence or absence of a PFO. On this day, an intravenous catheter (IV) was placed to draw venous blood to measure WBC counts and for the PFO screening. On the second day, an IV was placed in the participant's arm for additional venous blood draws. Venous blood was drawn to measure inflammatory cytokine concentrations and to measure HbCO during the Hb mass test. After the IV was placed, blood was drawn for baseline cytokine analysis. After the baseline blood draw, participants completed the Hb mass test.

Blood collection

All subjects in the present study participated in the Colorado Oregon Altitude Study (COAST); however, some subjects participated in other studies with various aims centered around the effects of PFO on cardiopulmonary physiology, oxygen transport, and the immune

system, named SWEAT and EPO. All three studies (COAST, SWEAT, and EPO) involved venous blood draws for cytokine analysis. COAST blood samples were taken on the same day as Hb mass testing prior to any CO inhalation. There was no control for time of day. SWEAT blood samples were taken on a separate day as Hb mass at approximately 7am, and EPO blood samples were taken on the same day as Hb mass at approximately 7am. Table 2 documents from which study baseline serum samples were used for cytokine analysis. If a subject had blood from multiple studies analyzed, those values were averaged.

Patent Foramen Ovale Screening

Saline contrast echocardiography is considered to be the most sensitive test for detection of intracardiac shunts as it assumes intravenously-infused saline contrast bubbles are either filtered by the pulmonary capillaries or transported through an intracardiac shunt such as a PFO (Lovering et al., 2012). To determine the presence or absence of a PFO, participants undergo a comprehensive ultrasound screening with saline contrast echocardiography, performed by a trained echocardiographer. The screening is performed with participants reclined on their left side, breathing ambient air. Participants are instrumented with an IV in a peripheral vein, and a bolus of manually agitated 3 ml sterile saline with 1 ml of ambient air is injected. Injections are performed during normal breathing and immediately following the release of a Valsalva maneuver. During the Valsalva maneuver, participants inhale and hold a small breath while contracting their abdominal muscles for roughly 10 seconds. Contraction during the Valsalva maneuver increases thoracic pressure and collapses veins. They are then asked to release this position. The release of the Valsalva maneuver results in a surge of blood flow, increasing right atrial pressure and allowing blood with the agitated saline to quickly travel across the shunt (Lovering et al., 2012). Blood flow across the PFO only occurs if the pressure gradient from the

right to left atrium is positive; therefore the purpose of the Valsalva maneuver is to increase this pressure gradient during screening. Ultrasound is used to visualize the heart and see if these microbubbles cross from the right atrium of the heart within 3 heart beats. If bubbles are present in the left heart within 3 heart beats, then this indicates a PFO (PFO+); if bubbles are absent from the left heart or appear after more than 3 cardiac cylces, then there is no PFO (PFO-).

Blood processing and analysis

Whole blood samples were collected to measure WBC count using anticoagulant ethylenediaminetetraacetic acid (EDTA) coated vacutainer tubes. Blood samples were kept at room temperature and sent to QUEST diagnostics within 48 hours for WBC count analysis with flow cytometry. Cytokines were measured from serum collected during venous blood draws with serum separator tubes. Whole blood was spun at 1500 g for 10 min, then serum was aliquoted into cryotubes and frozen at -80℃ until analysis. Cytokine analysis was performed using the BioLegend 13-plex human inflammation panel, according to manufacturer guidelines. Cytokines were analyzed via flow cytometry with an immunosorbent assay similar to an ELISA, using the V bottom plate protocol. Samples were removed from the freezer and fully thawed over ice. Samples were diluted 1:2, per user guidelines. The flow rate was set to low and the set number of beads to be acquired was 300 per analyte. All samples were measured in duplicate. Any sample with a coefficient variation (CV) greater than 30% was excluded from analysis per user guidelines.

Hemoglobin Mass Test

Hb mass data was collected using the 10-minute CO rebreathing technique, as others have previously done, with all measures done in duplicate and within a percent error of 3% or less to ensure accuracy (Thomsen et al., 1991; Burge & Skinner, 1995; Garvican et al., 2010;

Siebenmann et al., 2017). The purpose of a Hb mass test is to measure RBC mass using the increased attraction of Hb for CO by having subjects breathe a small volume of CO (1 ml/kg) and measuring the percent change from baseline in HbCO. The CO rebreathing method is a minimally invasive, effective way to look at blood volume and mass of circulating Hb in the body, and is therefore used frequently in the research settings (Schmidt & Prommer, 2005). There are only minor side effects associated with the test associated with elevated levels of CO, including but not limited to: confusion, headache, fatigue, nausea, shortness of breath, dizziness and cough. Participants voluntarily consent and are reminded of the potential side effects associated with increased CO levels prior to the test, and are asked to notify the researchers if they experience any symptoms.

Hb mass testing was not restricted by time of day. To begin, an IV was placed in the participant's arm and they were instructed to rest in the supine position quietly for 20 minutes. During this time, the CO syringe was set to the appropriate CO volume. This is determined by body weight, with 1 mL/kg of CO administered for males. This allows us to control for the percent of HbCO in each participant. HbCO should be kept between 10-15%. The circuit is also adjusted to fit the participant, ensuring comfort and correct positioning of mouthpiece. Within the last 5 minutes of supine rest, the closed circuit is flushed 3 times with 100% oxygen. After 20 minutes, the participant put on nose clips and began breathing on the mouthpiece. They began by breathing 100% oxygen for 4 minutes. At the end of 4 minutes, a 2mL blood sample was collected. The blood is analyzed using a co-oximeter (Copenhagen Radiometer OSM3) to look at the percent of CO bound to Hb. After the blood sample was taken, the participant was instructed to exhale completely and was switched to the rebreathing circuit. At this time, a 99.9% pure CO bolus was delivered slowly. A 10-minute timer was started once all CO had

been delivered. The rebreathing circuit was monitored continuously and filled with oxygen as needed on the subject's exhalation. After 10 minutes, another 2mL blood sample is drawn and run through the Copenhagen Radiometer OSM3 to look at changes in HbCO. After the blood draw, the participant exhaled completely and the rebreathe circuit was closed. The participant was then instructed to rest and breathe ambient air. The total volume of gas in circuit and concentration of CO remaining in circuit was measured. After the volume of gas and concentration of CO was measured, the rebreathe circuit is flushed with 100% oxygen to ensure no remaining CO in the circuit for the duplicate measure. Then, after 10 minutes of rest, the participant repeated the above procedure for a second measure. Supplemental O_2 was administered if the change in HbCO changes by more than 20% as a safety precaution. No participants reached had a change in HbCO greater than 20%.

Statistical Analysis

Alpha was set to 0.05, denoting a significant p value. All values are present as mean $\pm$ standard deviation. To determine differences in absolute Hb mass between those with and without a PFO an unpaired t test was used. To determine the difference in relative Hb mass between those with and without a PFO an unpaired t test with Welch's correction was used. In addition, an unpaired t test was used to determine differences between those with and without a PFO in both overall WBC count and individual WBCs (neutrophils, lymphocytes, monocytes, eosinophils, basophils). Lastly, an unpaired t test was used to determine differences between those with and without a PFO for the cytokines IL-6, IL-8, IL-12p70, IL-17A, IL-18, IL-23, IL-33, IFN-α2, IFN-γ, TNF-α and MCP-1. We used Welch's correction for cytokines IL-β and IL-10. The Welch's correction was applied to account for unequal standard deviations.

To determine whether there were relationships between cytokine concentrations and WBC counts with absolute and relative Hb mass, simple linear regression analyses were used. Simple linear regressions were also used to determine the relationships between the same variables in those with and without a PFO.

Results

Absolute/Relative Hb Mass in PFO+ and PFO- participants

There was no difference in absolute Hb mass between PFO- and PFO+ participants ($p = 0.1096$) (Figure 1A). Similarly, there was no differences in relative Hb mass between PFO- and PFO+ participants ($p = 0.1382$) (Figure 1B).

White Blood Cell Count/Individual White Blood Cells in PFO+ and PFO- participants

The was no difference in total WBC count between PFO- and PFO+ participants ($p = 0.8680$) (Figure 2A). There were also no differences in neutrophil count ($p = 0.5418$), lymphocyte count ($p = 0.6721$), monocyte count ($p = 0.5388$), eosinophil count ($p = 0.5603$), or basophil count ($p = 0.2539$) between PFO- and PFO+ participants (Figure 2B).

Cytokines in PFO+ and PFO- participants

There were no significant differences in the concentrations of IL-1β ($p = 0.3625$), IFN-α2 ($p = 0.5018$), IFN-γ ($p = 0.6285$), TNF-α ($p = 0.1760$), IL-6 ($p = 0.2077$), IL-8 ($p = 0.1412$), IL-10 ($p = 0.1231$), IL-12p70 ($p = 0.1270$), IL-17A ($p = 0.4687$), or IL-23 ($p = 0.3208$) between PFO- and PFO+ participants (Figure 3A). In addition, there were no differences in the concentration of MCP-1 ($p = 0.0577$), IL-33 ($p = 0.5131$), or IL-18 ($p = 0.4892$) between PFO- and PFO+ participants (Figure 3B).

Relationships between Cytokines and Hb Mass

Table 3 shows the slope, R^2, and p-value of the relationships between cytokine concentrations and WBC counts, and both absolute and relative Hb mass. There was a significant negative, linear relationship between IFN-α2 concentration and absolute Hb mass ($p = 0.0390$, $R^2 = 0.2158$, Figure 4 & Table 3). IL-6 and IL-10 were trending towards significant ($p = 0.0755$, $R^2 = 0.1842$; $p = 0.081$, $R^2 = 0.1782$, respectively) (Table 3). No other significant

relationships were found between the remaining cytokines and either absolute or relative Hb mass (Table 3). No relationships were found between any of the WBC counts and absolute or relative Hb mass (Table 3).

Relationship between Cytokines and Hb Mass for PFO+ and PFO- participants

Table 4 shows the slope, R^2, and p-value of the relationships between cytokine concentrations and WBC counts, and absolute and relative Hb mass for PFO- and PFO+ participants. There was a significant negative, linear relationship for PFO+ participants only between MCP-1 concentration and absolute Hb mass ($p = 0.0258$, $R^2 = 0.4823$, Figure 5A & Table 4). In addition, there was a significant negative, linear relationship for PFO+ participants only between IL-23 concentration and absolute Hb mass ($p = 0.0497$, $R^2 = 0.5003$, Figure 5B & Table 4). The relationship between monocytes and absolute Hb mass in PFO+ participants was trending towards significant ($p = 0.0768$, $R^2 = 0.3400$, Table 4). In addition, the relationship between monocytes and relative Hb mass in PFO+ participants was trending towards significant ($p = 0.0930$, $R^2 = 0.3124$, Table 4). No other relationships were found for the remaining cytokines or WBCs and absolute or relative Hb mass in PFO+ participants (Table 4).

There was a significant negative, linear relationship for PFO- participants only between IL-10 concentration and absolute Hb mass ($p = 0.0392$, $R^2 = 0.5350$, Figure 6 & Table 4). The relationship between IFN-α2 and absolute Hb mass was trending towards significant in PFO- participants ($p = 0.0865$, $R^2 = 0.3230$, Table 4). Similarly, the relationship between TNF-α and relative Hb mass in those without a PFO was trending towards significant ($p = 0.0824$, $R^2 = 0.3696$). No other relationships were found between the remaining cytokines or WBCs and absolute or relative Hb mass in PFO- participants (Table 4).

Tables

Participants	PFO	Age (years)	Weight (kg)	Height (cm)
20 males	10 PFO+ and 10 PFO-	26.2 ± 4.8	77.4 ± 9.5	180.7 ± 4.6

Table 1. Anthropomorphic data for study participants.

There were 20 total male participants, 10 with a PFO and 10 without a PFO. Data presented as mean ± standard deviation.

Subject ID	COAST	SWEAT	EPO
Subject 1		X	X
Subject 2	X		
Subject 3		X	
Subject 4	X		
Subject 5	X		
Subject 6	X		
Subject 7	X		
Subject 8	X		
Subject 9	X		
Subject 10	X		
Subject 11		X	
Subject 12		X	
Subject 13	X		
Subject 14		X	X
Subject 15	X		
Subject 16	X		
Subject 17	X		
Subject 18	X		
Subject 19		X	
Subject 20		X	

Table 2. Blood and Serum collections for study participants

Participants in our current study were also involved in other studies in our lab during this time. Blood and serum samples for the participants were collected through COAST, SWEAT and EPO data collection days. Subjects with blood from multiple studies were averaged.

	Absolute Hb mass			Relative Hb mass		
	Slope	**R^2**	**p-value**	**Slope**	**R^2**	**p-value**
IL-1β	-1.1660	0.1596	0.1252	0.0054	0.0252	0.5570
IFN-α2	-2.1160	0.2158	0.039 *	-0.0038	0.0059	0.7475
IFN-γ	-0.7278	0.1425	0.1654	0.0013	0.0021	0.8710
TNF-α	-0.3402	0.1777	0.0723	0.0013	0.0240	0.5270
MCP-1	-0.0891	0.0508	0.3395	-0.0012	0.0829	0.2182
IL-6	-1.0760	0.1842	0.0755 †	-0.0014	0.0025	0.8450
IL-8	-0.1916	0.0562	0.3285	0.0008	0.0085	0.7076
IL-10	-0.6207	0.1782	0.081 †	-0.0007	0.0017	0.8722
IL-12p70	-1.0510	0.1346	0.1475	-0.0032	0.0106	0.6941
IL-17A	-4.9870	0.0217	0.5725	-0.0135	0.0013	0.8912
IL-18	0.0629	0.0080	0.7075	0.0006	0.0065	0.7356
IL-23	-0.4230	0.1228	0.1678	-0.0002	0.0002	0.9559
IL-33	-0.0772	0.1190	0.1363	-0.0003	0.0136	0.6245
WBCs	-11.7800	0.0218	0.5346	0.0662	0.0059	0.7476
Neutrophils	-0.0062	0.0031	0.8170	0.0003	0.0599	0.2982
Lymphocytes	-0.0279	0.0248	0.5070	-0.0003	0.0204	0.5482
Eosinophils	-0.1393	0.0106	0.6653	-0.0033	0.0526	0.3306
Monocytes	-0.3515	0.1378	0.1071	-0.0025	0.0597	0.2994
Basophils	-0.0485	0.0000	0.9819	0.0053	0.0030	0.8184

Table 3. Relationships between cytokines/WBCs and absolute/relative Hb mass.

Table 3 shows the relationship between both cytokines and WBCs with absolute and relative Hb mass. Presented in the table is the slope, R^2, and p value for each relationship. Significant relationships presented with * ($p < 0.05$). Relationships trending towards significant † ($p < 0.10$).

	Absolute Hb mass					
	PFO-			PFO+		
	Slope	R^2	p-value	Slope	R^2	p-value
IL-1β	-0.6697	0.4048	0.1245	-2.3940	0.0896	0.4340
IFN-α2	-1.6000	0.3230	0.0865 †	-2.5690	0.1567	0.2574
IFN-γ	-0.3798	0.1843	0.3364	-1.2870	0.1958	0.2722
TNF-α	-0.2271	0.2578	0.1629	-1.1800	0.1623	0.2483
MCP-1	0.1096	0.1847	0.2152	-0.4852	0.4823	0.0258 *
IL-6	-0.8136	0.3373	0.1010	-5.2950	0.1590	0.2878
IL-8	-0.0892	0.0952	0.4191	0.5074	0.0205	0.6930
IL-10	-0.4009	0.5350	0.0392 *	-0.5499	0.0045	0.8534
IL-12p70	-0.5464	0.3791	0.1410	-2.9010	0.0323	0.6194
IL-17A	-4.4970	0.1272	0.3858	-14.8100	0.0978	0.4125
IL-18	0.0436	0.0068	0.8213	0.1651	0.0472	0.5465
IL-23	-0.1592	0.1300	0.3404	-3.6830	0.5003	0.0497 *
IL-33	-0.0542	0.1190	0.3289	-0.0797	0.0945	0.3877
WBCs	6.4520	0.0085	0.7997	-19.3200	0.0640	0.4807
Neutrophils	0.0026	0.0010	0.9317	-0.0027	0.0005	0.9506
Lymphocytes	0.0136	0.0116	0.7671	-0.0858	0.1867	0.2124
Eosinophils	0.7073	0.2128	0.1796	-0.4487	0.1420	0.2831
Monocytes	-0.1529	0.0279	0.6449	-0.5127	0.3400	0.0768 †
Basophils	-1.8960	0.0498	0.5354	2.1550	0.0611	0.4910

	Relative Hb mass					
	PFO-			PFO+		
	Slope	R^2	p-value	Slope	R^2	p-value
IL-1β	0.0091	0.4296	0.1100	0.0180	0.0339	0.6356
IFN-α2	0.0066	0.0714	0.4554	-0.0184	0.0586	0.5004
IFN-γ	0.0034	0.0885	0.5171	-0.0017	0.0010	0.9407
TNF-α	0.0022	0.3696	0.0824 †	0.0067	0.0384	0.5873
MCP-1	-0.0005	0.0545	0.5164	-0.0014	0.0280	0.6440
IL-6	0.0024	0.0413	0.6000	-0.0281	0.0321	0.6445
IL-8	0.0017	0.1814	0.2531	0.0122	0.0870	0.4080
IL-10	0.0019	0.0687	0.5306	0.0115	0.0145	0.7400
IL-12p70	0.0017	0.0169	0.7810	-0.0506	0.0718	0.4542
IL-17A	0.0682	0.1310	0.3783	-0.2048	0.1341	0.3324
IL-18	-0.0011	0.0532	0.5214	0.0030	0.1104	0.3483
IL-23	0.0023	0.1477	0.3071	-0.0337	0.2748	0.1823
IL-33	0.0003	0.0628	0.4849	-0.0007	0.0584	0.5013
WBCs	0.2710	0.1974	0.1983	-0.0198	0.0005	0.9515
Neutrophils	0.0004	0.2902	0.1082	0.0003	0.0573	0.5053
Lymphocytes	0.0001	0.0021	0.9007	-0.0007	0.0791	0.4312
Eosinophils	-0.0043	0.1047	0.3618	-0.0041	0.0860	0.4109
Monocytes	0.0037	0.2134	0.1790	-0.0057	0.3124	0.0930 †
Basophils	-0.0252	0.1158	0.3361	0.0337	0.1094	0.3505

Table 4. Relationships between cytokines/WBCs and absolute/relative Hb mass for PFO+ and PFO- participants.

Table 4 shows the relationship between both cytokines and WBCs with absolute and relative Hb mass. Presented in the table is the slope, R^2, and p value for each relationship. Significant relationships presented with * ($p < 0.05$). Relationships trending towards significant † ($p < 0.10$).

Figures

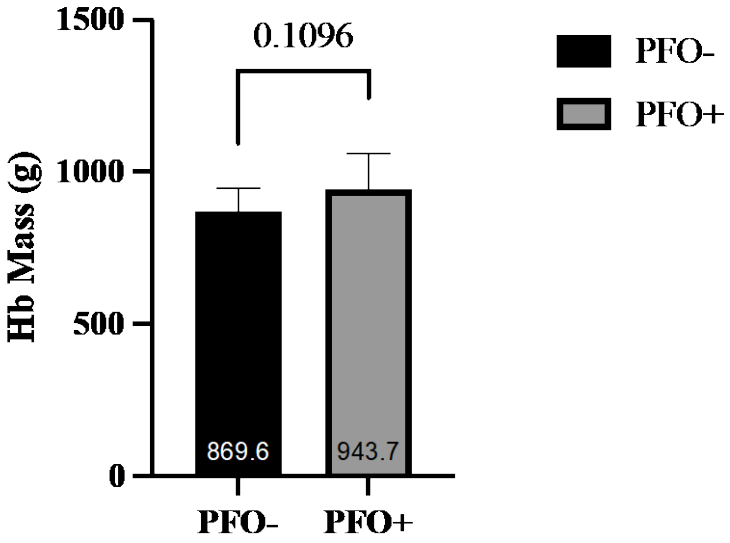

Figure 1A. Absolute Hb Mass (g) in PFO+ and PFO- Subjects

Figure 1A shows the group mean differences for absolute Hb mass between those with and without a PFO. **Figure 1B** shows the group mean differences for relative Hb mass between those with and without a PFO. Those with a PFO are illustrated in gray, while those without a PFO are shown in black. The graph bars indicate the average Hb mass for PFO+ and PFO- participants. The average values of Hb mass are also presented inside the corresponding bars. The p value shown above the graph indicates no statistical difference between PFO+ and PFO- participants when looking at absolute or relative Hb mass. Data are mean $\pm$ standard deviation.

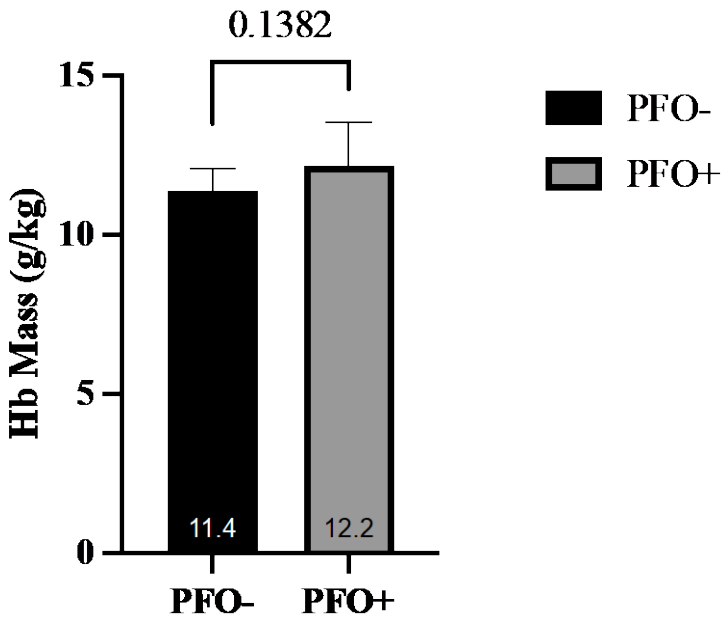

Figure 1B. Relative Hb Mass (g/kg) in PFO+ and PFO- Subjects

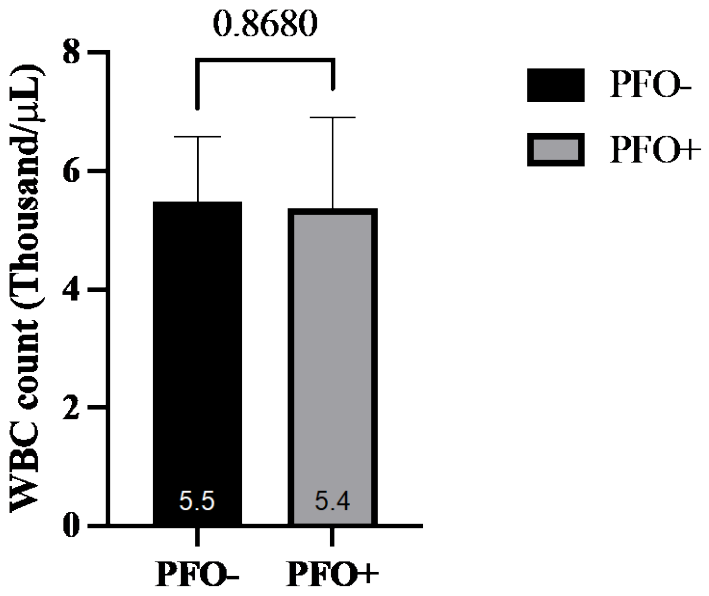

Figure 2A. WBC Count (Thousand/μL) in PFO+ and PFO- Subjects

Graph shows the relationship between the presence or absence of a PFO on WBC count. Those with a PFO are illustrated in gray, while those without a PFO are shown in black. The graph bars indicate the average WBC count for PFO+ and PFO- participants. The average values of WBC count are also presented inside the corresponding bars. The p value shown above the graph indicates no statistical difference between PFO+ and PFO- participants when looking at WBC count (p = 0.8680). Data are mean ± standard deviation.

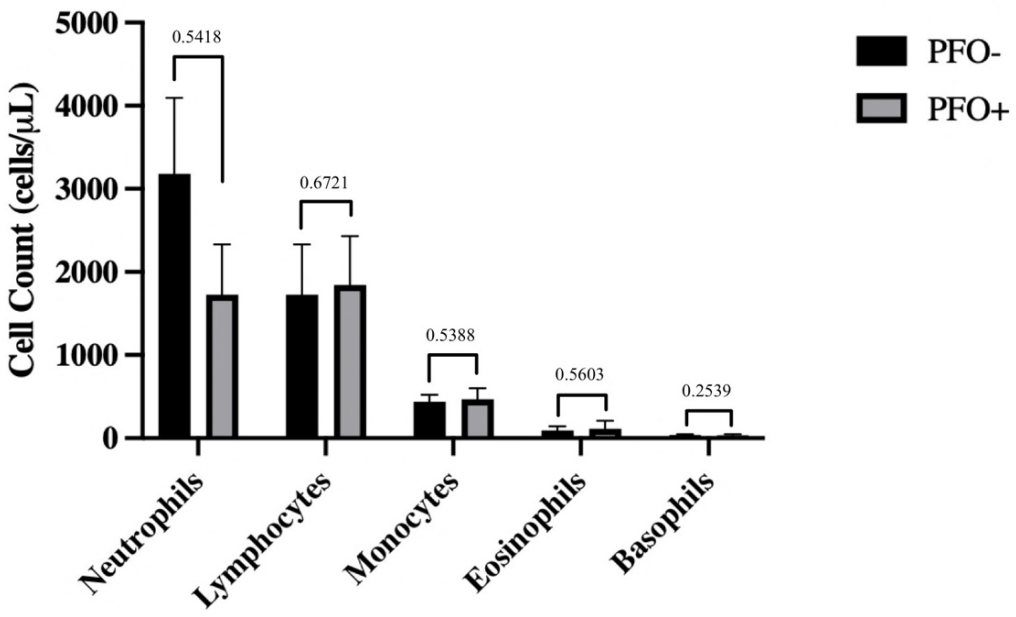

Figure 2B. Individual WBC Counts (cells/μL) in PFO+ and PFO- Subjects

Graph shows the relationship between the presence or absence of a PFO on individual WBCs; neutrophils, lymphocytes, monocytes, eosinophils, basophils. Those with a PFO are illustrated in gray, while those without a PFO are shown in black. The graph bars indicate the average WBC count for PFO+ and PFO- participants. The p values are shown over each WBC and indicate no statistical difference between PFO+ and PFO- participants when looking at individual WBCs (p = 0.5418, p = 0.6721, p = 0.5388, p = 0.5603, p = 0.2539, respectively). Data are mean ± standard deviation.

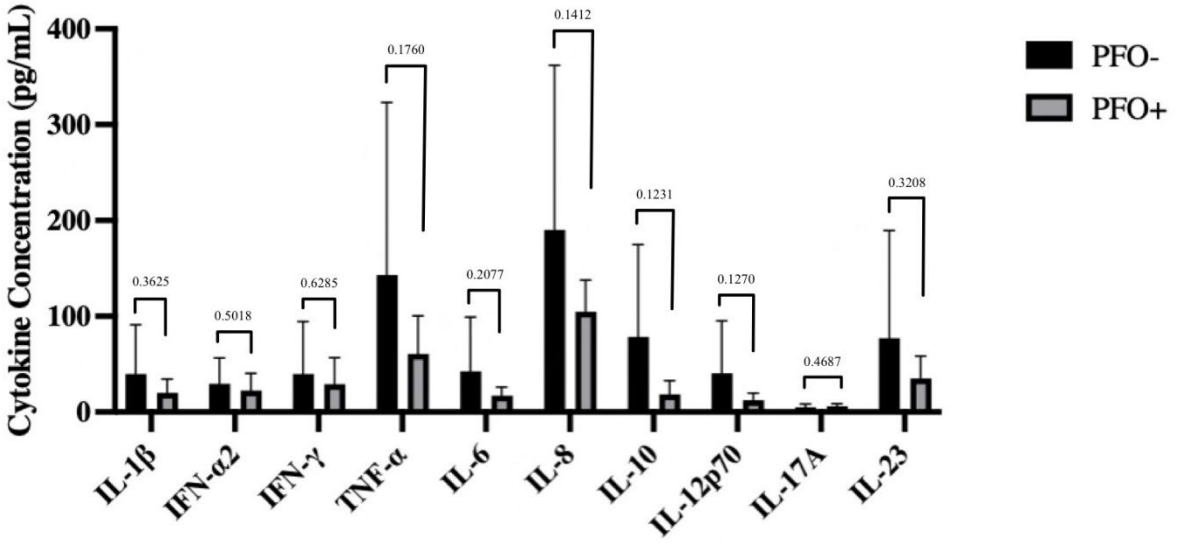

Figure 3A. Cytokine Concentrations (pg/mL) in PFO+ and PFO- Subjects

Graph shows the relationship between the presence or absence of a PFO on cytokine concentration for IL-β1, IFN-α2, IFN-γ, TNF-α, IL-6, IL-8, IL-10, IL-12p70, IL-17A and IL-23. Those with a PFO are illustrated in gray, while those without a PFO are shown in black. The graph bars indicate the average cytokine concentration for PFO+ and PFO- participants. The p values are shown over each cytokine and indicate no statistical difference between PFO+ and PFO- participants when looking at these specific cytokines (p = 0.3625, p = 0.5018, p = 0.6285, p = 0.1760, p = 0.2077, p = 0.1412, p = 0.1231, p = 0.1270, p = 0.4687, p = 0.3208, respectively). Data are mean $\pm$ standard deviation.

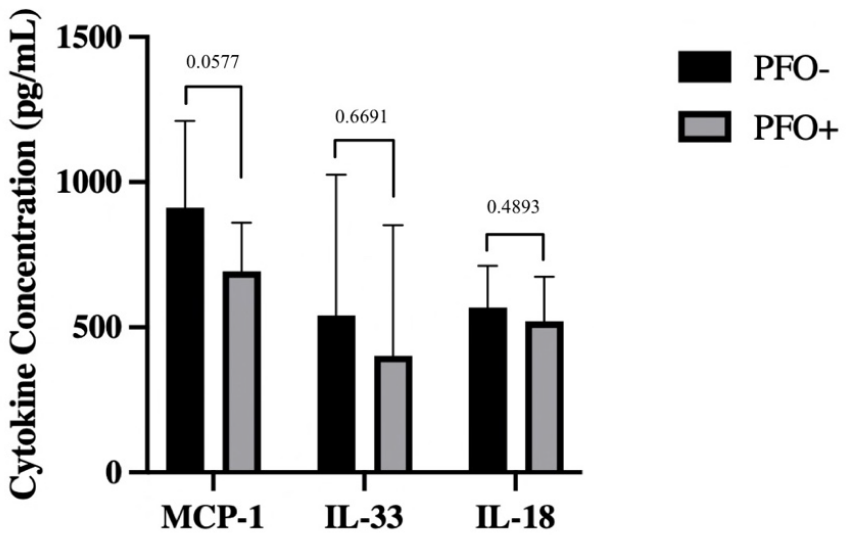

Figure 3B. Cytokine Concentrations (pg/mL) in PFO+ and PFO- Subjects

Graph shows the relationship between the presence or absence of a PFO on cytokine concentration for MCP-1, IL-33 and IL-18. Those with a PFO are illustrated in gray, while those without a PFO are shown in black. The graph bars indicate the average cytokine concentration for PFO+ and PFO- participants. The p values are shown over each cytokine and indicate no statistical difference between PFO+ and PFO- participants when looking at these specific cytokines ($p = 0.0577$, $p = 0.6691$, $p = 0.4893$, respectively). Data are mean $\pm$ standard deviation.

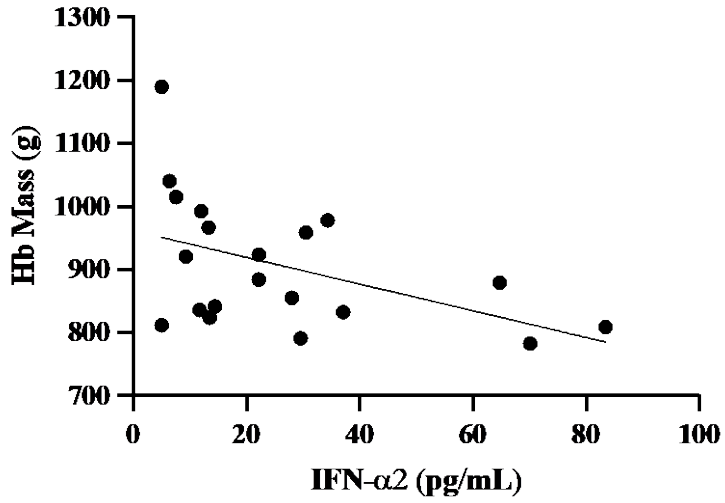

Figure 4. Relationship between IFN-α2 (pg/mL) and Absolute Hb Mass (g)

Graph shows the relationship between IFN-α2 concentration and absolute Hb mass for our whole group data. The individual data points show the concentration of IFN-α2 and absolute Hb mass for each participant in this study. The trend shows a negative, linear relationship between IFN-α2 and absolute Hb mass ($R^2 = 0.2158$, $p = 0.039$).

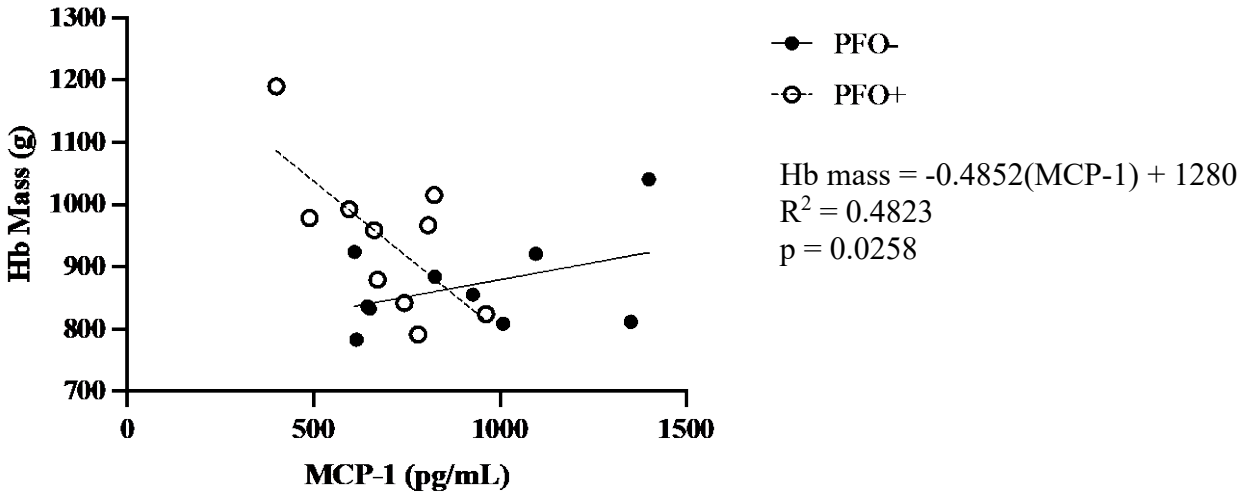

Figure 5A. Relationship between MCP-1 (pg/mL) and Absolute Hb Mass (g) for PFO+

Graph shows the relationship between MCP-1 concentration and absolute Hb mass for those with a PFO. Those with a PFO are shown with open circles, while those without a PFO are shown with filled in circles. The dotted trend line shows the relationship for those with a PFO, while the solid trend line shows the relationship for those without a PFO. The individual data points show the concentration of MCP-1 and absolute Hb mass. The trend shows a negative, linear relationship between MCP-1 and absolute Hb mass ($R^2 = 0.4823$, $p = 0.0258$).

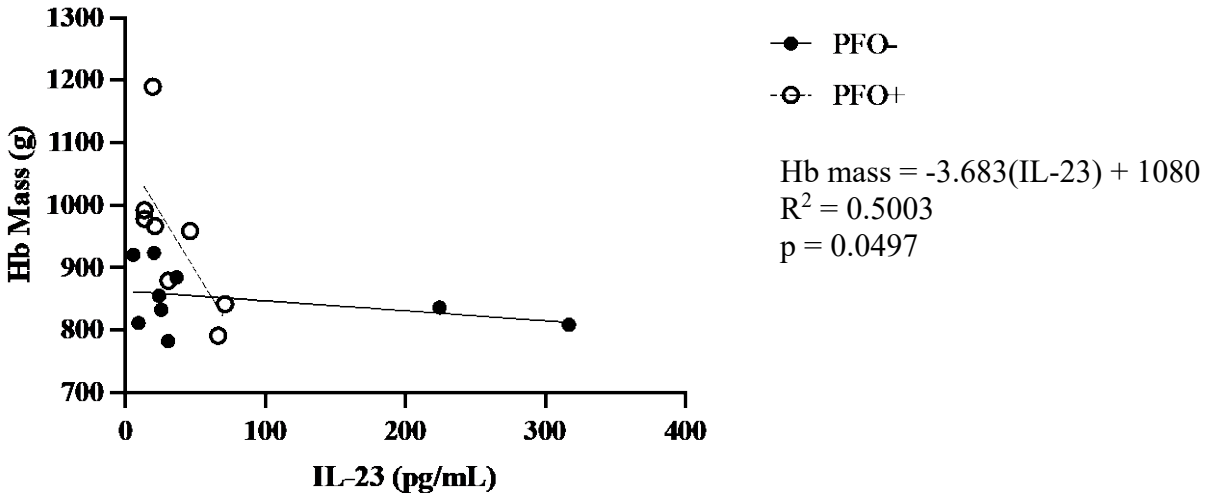

Figure 5B. Relationship between IL-23 (pg/mL) and Absolute Hb Mass (g) for PFO+

Graph shows the relationship between IL-23 concentration and absolute Hb mass for those with a PFO. Those with a PFO are shown with open circles, while those without a PFO are shown with filled in circles. The dotted trend line shows the relationship for those with a PFO, while the solid trend line shows the relationship for those without a PFO. The individual data points show the concentration of IL-23 and absolute Hb mass. The trend shows a negative, linear relationship between IL-23 and absolute Hb mass ($R^2 = 0.5003$, $p = 0.0497$).

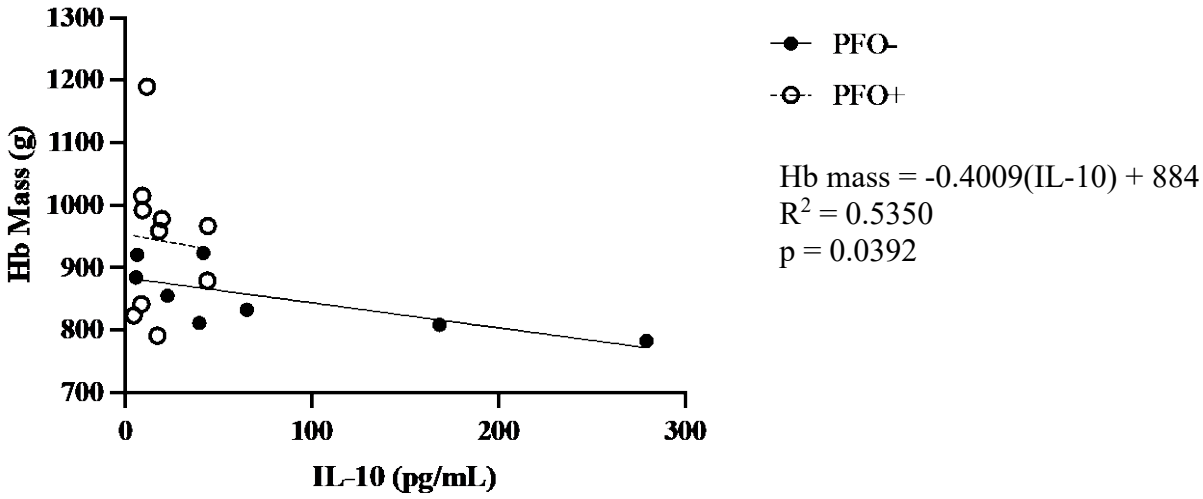

Figure 6. Relationship between IL-10 (pg/mL) and Absolute Hb Mass (g) for PFO-

Graph shows the relationship between IL-10 concentration and absolute Hb mass for those without a PFO. Those with a PFO are shown with open circles, while those without a PFO are shown with filled in circles. The dotted trend line shows the relationship for those with a PFO, while the solid trend line shows the relationship for those without a PFO. The individual data points show the concentration of IL-10 and absolute Hb mass. The trend shows a negative, linear relationship between IL-10 and absolute Hb mass ($R^2 = 0.5350$, $p = 0.0392$).

Discussion

The purpose of this study was to investigate the relationships between RBC mass and the presence of a PFO, inflammatory cytokine concentrations, and WBCs. We hypothesized that those with a PFO would have both a higher WBC count and cytokine concentrations, leading to a decreased Hb mass.

Presence of a PFO and Hb Mass

No differences were found for absolute (g) ($p = 0.1096$) or relative (g/kg body weight) ($p = 0.1382$) Hb mass between PFO+ and PFO- participants. From this, we can conclude that the presence of a PFO alone is not expected to impact Hb mass. We hypothesized that a PFO might impact Hb in two ways. First, those with a PFO have the potential to have an increase in arterial hypoxemia given that a PFO allows some deoxygenated blood to enter systemic circulation. In this case, arterial hypoxemia could result in a decrease in oxygen delivery to the kidney, stimulating an increase in erythropoiesis and increasing Hb mass. This was taken into consideration due to the findings in a study conducted by Lovering *et al.* (2011), in which PFO+ subjects had lower PaO2 at rest and had worse resting pulmonary gas exchange efficiency. However, in another study conducted by Duke *et al.* (2020), no differences were found between those with and without a PFO in arterial hypoxemia at sea level despite those with a PFO+ having worse gas exchange efficiency. Given this discrepancy and the minimal published PFO research, it is unknown whether the PFO drives arterial hypoxemia and if this impacts Hb mass. Therefore, it may be worth considering potential size or sex differences when looking at the relationship between PFO and Hb mass. Preliminary data from our lab suggests that women with a large PFO have worse pulmonary gas exchange efficiency during exercise, while men with a PFO do not have worse pulmonary gas exchange efficiency during exercise. The second

way in which a PFO was hypothesized to impact Hb mass was through altering cytokine and WBC expression, which will be discussed more below.

Presence of a PFO and WBC count, Individual WBCs and Cytokine Concentrations

No differences were found for WBC count ($p = 0.8680$) between PFO+ and PFO- participants and for each of the respective immune cells; neutrophils ($p = 0.5418$), lymphocytes ($p = 0.6721$), monocytes ($p = 0.5388$), eosinophils ($p = 0.5603$), basophils ($p = 0.2539$). There were also no inflammatory differences detected for any of the cytokines for PFO+ and PFO- participants, contradicting our preliminary data. We hypothesized that those with a PFO would have higher cytokine concentrations and WBC counts, which we did not observe.

As mentioned above, the lack of significant differences in cytokine concentrations between those with and without a PFO contradicts the preliminary data previously found in our lab. Inconsistency between our results may be due to individual variability and a wide range of observed values in both the previous study and our current study. For example, the high standard deviations in the current study were largely driven by a couple PFO- participants who had consistently high levels of cytokines that may have skewed our results. In addition, there was great variability in cytokine detection levels, ranging from levels well below detection up to very high concentrations. It is important to consider that both our preliminary data and our current study had relatively small sample sizes. In addition, the present study did not aim to change inflammation. Therefore, an interventional study that aims to alter inflammation would be a potentially new area for investoagtion when looking at differences in cytokine concentrations. Given that no other studies have looked at inflammation in those with and without a PFO, more

research must be conducted to determine if there are significant and physiologically meaningful differences between those with and othout a PFO.

Associations between Hb Mass, WBC Count and Cytokine Concentration

There were no significant relationships between WBC count and either absolute or relative Hb mass. Importantly, while WBC count was measured within a short time period of the participants' Hb mass tests, these measurements were not done on the same day. More time between these measures allows for more variability and potential changes in immune cell expression. Therefore, if a subject was experiencing any initial symptoms of an infection on either the day that WBC counts were measured or Hb mass was measured, for example, it may alter the relationships between these variables. We also did not look directly at macrophages, which play an important role in modulating RBC regulation through recycling RBCs. Therefore, it may be worth focusing on specific WBCs, such as macrophages, when investigating the effects of immune cells on Hb mass.

There was a significant and negative linear relationship between IFN-α2 and absolute Hb mass ($p = 0.0390$, $R^2 = 0.2158$). Other studies, not conducted in humans, have shown that IFN-α2 inhibits erythroid progenitors and can induce programmed cell death of these cells (Lai et al., 1995; Tarumi et al., 1995). Our data supports these findings in a human model because increased levels of IFN-α2 are associated with a decrease in Hb mass. This decrease in Hb mass may be due to the inhibition or cell death of erythroid progenitors from IFN-α2. In regards to the other cytokines, no significant relationships were found between cytokine concentration and Hb mass. This may be due to individual variability, outliers, small sample sizes, or these cytokines having a limited role in Hb mass regulation in humans.

Hb Mass and Cytokine Concentrations in those with a PFO

There was a significant and negative linear relationship between MCP-1 concentration and absolute Hb mass in PFO+ participants ($p = 0.0258$, $R^2 = 0.4823$), but not in PFO- participants. Similarly, there was a relationship between IL-23 levels and absolute Hb mass in PFO+ participants ($p = 0.0497$, $R^2 = 0.5003$), but not in PFO- participants. No significant relationships were detected between the remaining cytokines and both absolute and relative Hb mass in PFO+ participants. Although there were no PFO differences in Hb mass, WBC counts or cytokine concentrations, the presence of a PFO may alter the relationship between Hb mass and the cytokines MCP-1, IL-23 and IL-10. No current studies have looked at the effect of MCP-1 on erythropoiesis. Therefore, our data is potentially the first to look at this relationship, but it is possible that other studies have not found anything because they have not considered the presence or absence of a PFO. In addition, the relationship between IL-23 and RBC regulation is not well understood. However, some studies suggest that IL-23 can interact with other cytokines such as TNF-α and IL-17 (Pastor-Fernández, 2020). We know that TNF-α has been shown to inhibit EPO, so IL-23 may have some indirect impacts on erythropoiesis through its interaction with other cytokines (Johnson et al., 1990; Fandrey & Jelkmann, 1991; Faquin et al., 1992; La Ferla et al., 2002). Although we are looking at these specific cytokines in this study, cytokines are rarely released alone and tend to interact with their environment and each other. Despite the lack of literature on MCP-1 and IL-23 directly, cytokines share many similar functions, and it is possible that MCP-1 and IL-23 can increase the activity of other cytokines that may impact erythropoiesis.

Hb Mass and Cytokine Concentration in those without a PFO

There was a significant and negative linear relationship between IL-10 levels and absolute Hb mass in PFO- participants ($p = 0.0392$, $R^2 = 0.5350$), but not in PFO+ participants. No significant relationships were observed between the other cytokines and either absolute or relative Hb mass in PFO- participants. IL-10 is an anti-inflammatory cytokine that acts to inhibit the production of other cytokines and immune cells. While the anti-inflammatory effects of IL-10 are well known, the data regarding its impact on erythropoiesis is not. Some studies have shown that in the presence of EPO, IL-10 can promote erythropoiesis (Rennick et al., 1994; Wang et al., 1996). However, in other studies, IL-10 has been shown to inhibit the formation of erythroid progenitor colonies (Oehler et al., 1999). The inhibition of erythroid progenitor colonies may lead to a decrease in erythropoiesis because less cells are committed to becoming mature RBCs. With a decrease in RBCs, there may also be a decrease in Hb mass. Despite the inconsistency in literature and this potential stimulatory role of IL-10, we still see lower Hb mass at higher IL-10 concentrations in those without a PFO.

Limitations

A limitation to this study is that we only looked at the relationships between RBC mass and the presence of a PFO, inflammatory cytokine concentrations, and WBCs in males. The female population was not considered in this study. This is an important limitation because the physiology between males and females can vary, particularly with respect to Hb levels. In females, sex hormones associated with the menstrual cycle can impact erythropoiesis and therefore RBC mass (Reeves et al., 2001; Coronado Daza & Cuchi, 2019). While there is no difference in basal EPO concentration, it is known that men have a higher mean Hb level (Murphy, 2014). Thus, these results cannot be conclusively applied to the female population because women were not directly tested in these studies. Another limitation to this study was our sample size. In this study, we looked at 20 healthy men, 10 with a PFO and 10 without a PFO. Due to our small sample size, it is not possible to attribute these findings to the entire population of those with and without a PFO. A larger group of individuals must be studied to better understand the impact of a PFO on variables such as cytokine concentrations, WBCs, and Hb mass. In addition, the differences between studies showing the impact of inflammation on RBC regulation and the present results may be due to the fact that the aim of this study was not to provide an intervention or stimulus to alter inflammation. Much of the data on the relationship between the immune system and RBC regulation is from interventional studies that aim to increase inflammation, and this was not the case in our study. In addition, these interventional studies are frequently conducted in animal models, unlike our study that utilized human subjects. To better understand the baseline levels of, and roles of circulating inflammation in, those with and without a PFO, and the impact of said inflammation on RBC mass, future studies should take an interventional approach.

Conclusion

Our preliminary data suggested that those with a PFO had higher levels of inflammatory cytokines in their blood compared to those without a PFO. Knowing that WBCs are largely responsible for producing cytokines, it was hypothesized that those with a PFO would have an increase in both WBC count and cytokine concentration, leading to a decrease in Hb mass. Contrary to our preliminary data and hypotheses, we found no differences in WBC count, cytokine concentration or Hb mass in those with or without a PFO, suggesting a PFO alone does not impact these variables in men. We did find a negative linear relationship between IFN-α2 and absolute Hb mass, indicating a potential inhibitory role this cytokine has on Hb mass regulation; however, no other relationships were found between WBCs or the other cytokines and Hb mass. Additionally, there was a negative linear relationship for both MCP-1 and IL-23 with absolute Hb mass in men with a PFO. There was a relationship between IL-10 and absolute Hb mass in men without a PFO. These findings suggest that the presence of a PFO may alter the relationship between Hb mass and the cytokines MCP-1, IL-23 and IL-10. Given the minimal literature on MCP-1 and IL-23 when it comes to erythropoeisis, it is possible that our data is one of the first to suggest a potential relationship, while also taking into consideration the PFO. These findings encourage further exploration to see how specific cytokines impact Hb mass both in the presence and absence of a PFO. To conclude, more research in this area must be conducted to better understand inflammation in those with and without a PFO, and it may be worth focusing on additional cytokines and specific WBCs when investigating the effect of immune cells on Hb mass. Understanding this relationship between the immune system and Hb mass may explain how inflammatory cytokines and WBCs regulate the production and survival of RBCs, both in those with and without a PFO.

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