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Extramedullary Stress Erythropoiesis as an Adaptive Response to High-Altitude Hypoxemia

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by

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ABSTRACT OF THE THESIS

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by

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Dr. Erica Heinrich, Chairperson

Systemic inflammation has been observed in sojourners traveling acutely to high-altitude. While chronic systemic inflammation is often associated with anemia, an alternative mechanism of extramedullary erythropoiesis, aptly named Stress Erythropoiesis (SE), has been shown in murine models to become activated in response to acute inflammatory and hypoxic stress. SE seems to play a protective role against inflammation-induced decreases in red blood cell production. While the mechanisms of SE are unclear in humans, it is likely that a similar protective response is activated in response to acute inflammation and hypoxic stress, such as in sepsis, acute lung injury, or travel to high-altitude. Therefore, the goal of my research was to identify and measure biomarkers of SE in high-altitude sojourners. I investigated Fetal Hemoglobin (HbF), Heme-Oxygenase 1 (HO-1), and Carbon Monoxide (CO) as potential candidate biomarkers of SE. Biomarkers were measured in peripheral blood of 15 participants at sea-level and high-altitude. Expression of *HBG1*, one of the genes that codes for the

characteristic gamma (ɣ) subunit of HbF, was significantly upregulated on day 3 at high-altitude ($p < 0.01$). Moreover, physiological assessments of participants on day 1 at high-altitude showed a significant increase in exhaled CO concentration when compared to measurements taken at sea-level ($t(28) = -2.57$, $p = 0.01$). Conversely, analysis of RNA sequencing of the gene coding for HO-1, *HMOX1*, resulted in a significant downregulation of HO-1 at high-altitude ($p <0.01$). Additionally, differential expression of proteomic output reveals other potential biomarkers of SE, such as AXIN1 which serves an important role in the regulation of Wnt signaling and SE. These findings provide support for potential activation of SE during acute high-altitude exposure and provide suggest an important role of hypoxia-induced inflammation in regulating erythropoiesis during acclimatization.

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INTRODUCTION

Oxygen Capture and Delivery

The efficient uptake of atmospheric oxygen and its subsequent delivery to tissues is a delicate, yet robust dynamic system. This process begins in the lungs, where oxygen inhaled from the ambient air moves by bulk flow from the pharynx, through the trachea, and into the bronchial tubes. At the ends of these tubes, oxygen diffuses into alveoli, which act as the final interface between the lung and the circulating blood. Here, oxygen diffuses passively down its partial pressure gradient through the pulmonary capillaries into the blood, where a majority of oxygen is transported bound to hemoglobin in red blood cells (RBCs).

Hemoglobin is a tetramer composed of two pairs of alpha (α) and beta (β) subunits, each of which can bind an oxygen molecule. The kinetics of the hemoglobin molecule allow for it to shift its affinity for oxygen in the presence of certain physiological and metabolic adjustment. For example, decreased pH leads to a decrease in hemoglobin's affinity for oxygen, allowing it to be released more readily at the tissues (Bellingham, Detter, and Lenfant 1971). Alternatively, hemoglobin with a lower p50 (the oxygen partial pressure at which hemoglobin is 50% saturated with oxygen) binds oxygen with a higher affinity. This flexibility allows hemoglobin to more effectively capture oxygen at the lung (Haldane effect) and deposit oxygen at the tissue (Bohr effect) where it can be used for a variety of mechanisms including cellular metabolism (Malte and Lykkeboe 2018; Klocke 1973). Since RBCs contain around 27 million hemoglobin

molecules, each capable of carrying 4 oxygen molecules, hemoglobin is a highly effective system for delivering large amounts of oxygen to tissues (D'Alessandro et al. 2017). In fact, without hemoglobin, the amount of dissolved oxygen in blood would be insufficient to support metabolic demands.

Steady-State Erythropoiesis

To maintain efficient hemoglobin activity, mature RBCs, or erythrocytes, maintain a pH around 0.2 more alkaline than circulating blood(Romero and Romero 2004). For this reason, aging erythrocytes are unable to maintain long-term membrane stability, with the average life span of these cells being about 120 days (Ganz 2012). Senescent RBCs are absorbed by erythrocyte-recycling macrophages derived from the liver and spleen, allowing the heme of the aged RBC to be reused in the production of newer RBCs. This mechanism of controlled RBC degradation is coupled with an equally dynamic production of new erythrocytes, a process called erythropoiesis.

Steady State Erythropoiesis (SSE) is the main mechanism of erythrocyte production and is localized in the bone marrow. At this site, hematopoietic stem cells (HSCs) differentiate into erythroid progenitor cells in the presence of interleukin-3 (IL-3), colony stimulating factor (CSF), and thrombopoietin (TPO) (Rongvaux et al. 2011; Willinger et al. 2011). It is at this point where the hemoglobin synthesis begins with the introduction of erythropoietin (EPO), a glycoprotein hormone produced in the kidneys which stimulates RBC production. These progenitor cells, now erythroblasts, will

continue to produce hemoglobin, as well as begin to discard their nucleus and other organelles. Erythrocyte-recycling macrophages aid the maturation of erythroblasts by providing the cells with iron derived from heme as well as phagocytizing the expelled organelles and nuclei (Bessis and Breton-Gorius 1962). Once hemoglobin is densely packed into the cells, the newly formed reticulocytes, are prepared to be released into circulation, where they will shed their remaining vestigial organelles and fully mature into erythrocytes. This process of replenishment serves as a dynamic counterbalance for the degradation of senescent RBCs. On average, the SSE mechanism is capable of outputting around 2.5×10^6 RBCs per second (Palis 2014).

Anemia of Inflammation

As discussed previously, SSE coupled with regulated erythrocyte recycling is sufficient to address the need for oxygen uptake and delivery during homeostasis. However, this balance become threatened with the introduction of inflammatory stress or oxygen limitation (hypoxia). Inflammation is an immune response mediated through the introduction of inflammatory cytokines at the site of infection or damage within the body. Constant activation of inflammatory pathways due to disease or autoimmune action results in anemia, or insufficient RBC production which limits the rate of oxygen delivery. This anemia of inflammation functions through the mechanisms of iron restriction, suppression of erythropoiesis, and reduced erythrocyte survival (Weiss, Ganz, and Goodnough 2019).

Iron absorption from both dietary and endogenous sources is mediated by ferroportin 1 (FPN1), the only known cellular iron exporting protein. During inflammation, the liver secretes hepcidin, which binds to FPN1 causing the internalization of the protein and effective halting of intercellular iron delivery into the plasma (Nemeth, Tuttle, et al. 2004). The upregulation of hepcidin has been found, in animal models, to be a result of the expression of the inflammatory cytokine interleukin 6 (IL-6) (Nemeth, Rivera, et al. 2004). The binding of IL-6 to its associated IL-6 receptor protein results in the formation of signal transducer and activator 3 (STAT3) transcription factor through the upregulation of the JAK tyrosine kinase family of proteins. STAT3 binds to the promoter region of the hepcidin gene and increases its expression (Muckenthaler et al. 2017). Ultimately, these cascading processes result in a retention of iron within the cells, further reducing the availability of iron in the plasma for erythropoiesis.

Erythropoietic capacity is further impaired during chronic inflammation through the downregulation of EPO. It was found that the inflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor (TNF) caused damage to EPO-producing epithelial cells in the kidney as well as inhibition of GATA binding factor 2 (GATA2) and hepatocyte nuclear factor 4 (HNF4), two key transcription factors in the regulation of the *EPO* gene (Jelkmann 2011; la Ferla et al. 2002). Additionally, the presence of circulating IL-1 and IL-6 was found to have an inhibitory effect on the binding affinity of EPO to its associated EPO receptor protein (Okonko et al. 2013). The reduction of EPO directly impacts erythropoiesis by inhibiting erythroid progenitor proliferation and differentiation

into mature erythrocytes. Additionally, the upregulation of inflammatory cytokines shift hematopoiesis in the bone marrow to favor the myeloid lineage (myelopoiesis). This has been observed as a result of induction of the Batf2 and C/EBPβ transcription factors interferon gamma (IFN-ɣ) receptor-expressing HSCs (Matatall et al. 2014).

In addition to impeding erythrocyte production, anemia of inflammation also contributes to increased erythrophagocytosis. In mice, induction of IFN-ɣ increases phagocytotic capacity in the spleen (Libregts et al. 2011). This increase in macrophage activity is thought to be a result of a larger deposition of RBC complement and antibodies in circulation (Moldawer et al. 1989).

Stress Erythropoiesis

To combat the detrimental consequences anemia of inflammation has on erythrocyte production and viability, a protective secondary erythropoietic mechanism is activated to prevent extreme reductions in RBC levels and tissue hypoxia. Originally observed using a mouse model of phenylhydrazine-induced anemia(Hara and Ogawa 1976), the mechanism of stress erythropoiesis (SE) occurs primarily in the spleen, in contrast to steady state erythropoiesis which occurs in the bone marrow and is induced by inflammatory cytokines present during acute inflammation (Figure 1). This compartmentalization is in part due to the abundance of bone morphogenic factor 4 (BMP4) in the spleen (Figure 2). BMP4 is expressed during acute anemia through the induction of the transcription factor SPI-C, toll like receptor (TLR) signaling, and Sonic

hedgehog ligand signaling as a means to upregulate cytokine production (Bennett et al. 2019; Harandi et al. 2010). Moreover, BMP4 signaling has been linked to the rapid proliferation of erythroid progenitors localized in the spleen (Lenox, Perry, and Paulson 2005).

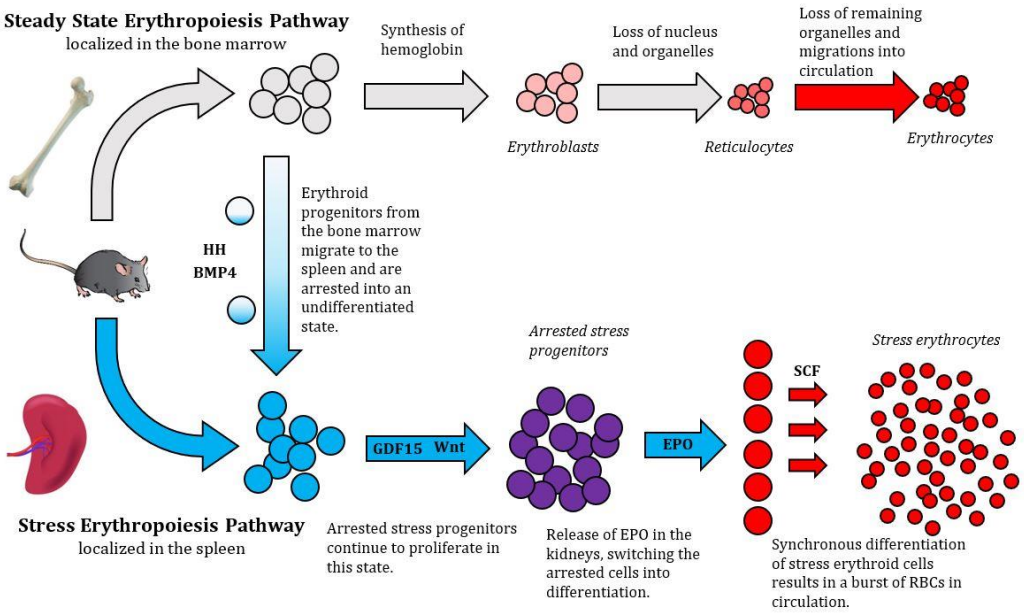

Figure 1. **Steady State Erythropoiesis vs Stress Erythropoiesis.** An overview of the stress and steady-state erythropoiesis mechanisms in mice. Steady-state erythropoiesis occurs in the bone marrow, while stress erythropoiesis occurs primarily in the spleen (Ji 2020). During normoxic conditions, hematopoietic stem cells produced in the bone marrow progress through the steady state erythropoietic pathway, resulting in the generation of hemoglobin and eventual loss of organelles and genetic material. During events of hypoxia and inflammation, these progenitors are arrested in an undifferentiated state and migrate to the spleen in the presence of HH and BMP4, where they will proliferate. In the spleen, these cells will switch from amplification to differentiation during a flood of EPO. Differentiated cells, known as stress erythrocytes, proliferate in a media of BMP4, SCF, and GDF15 and are released into circulation in response to hypoxic stress (Azzara 2017).

These newly formed stressed erythroid progenitors (SEPs) possess many of the same cell surface proteins as progenitors derived from the bone marrow, however, only the former is capable of responding to BMP4 (Akashi et al. 2000). Following the migration to the spleen, SEPs then commit to a lineage characterized by the expression of the immature cell marker proteins, Kit, CD34, CD133, and Sca1 (Xiang et al. 2015; Perry, Harandi, and Paulson 2007; Harandi et al. 2010). After committing to a cell lineage, SEPs are arrested in an undifferentiated state and begin proliferating at a rapid rate, mediated by the cultivation of a splenic niche consisting of growth factor differentiation 15 (Gdf15) and Wnt signaling (Y. Chen et al. 2020; Hao et al. 2019). Additionally, the increased rate of myelopoiesis, contributes to an increasing population of macrophages localized in the spleen, which serve as crucial players in the stress erythroid niche (Pietras et al. 2016; Liao, Prabhu, and Paulson 2018; Pietras 2017; Y. Chen et al. 2020). This synchronous amplification illustrates the characteristic “burst” of erythrocytes produced through this mechanism as a means to compensate for the lack of circulating erythrocytes.

Following proliferation, SEPs transition into differentiation into erythrocytes through the induction of Epo signaling in the splenic niche. It is important to note that Epo does not interact directly with SEPs, rather through altering macrophage signaling within the splenic niche. This is done through the downregulation of Wnt-expression proteins and upregulation of prostaglandins Pgj_2 and Pge_2 (Y. Chen et al. 2020; Xiang et al. 2015). This shift halts the proliferation of SEPs and promotes their differentiation into erythrocytes through the expression of Epo receptors. Upon differentiation, the bolus of

newly formed RBCs is released into circulation. This extramedullary mechanism of SE has been observed in humans in cases of anemia, however the localization of this process has not yet been determined (Orphanidou-Vlachou, Tziakouri-Shiakalli, and Georgiades 2014; Hara and Ogawa 1976).

Heme Recycling

Mice exposed to inflammatory stress undergo a slightly divergent mechanism of heme recycling as a result of SE pathway activation compared to steady-state heme recycling mechanisms. As mentioned previously, senescent erythrocytes are routinely recycled by hepatic macrophages to recycle heme. This recycling of endogenous heme constitutes the primary source of available iron (around 24-25mg daily) for erythropoiesis as dietary iron intake (1-2 mg daily) alone cannot meet the demand for homeostasis (Hentze et al. 2010). In cases of inflammatory stress, an increase in myelopoiesis as well as the larger quantity of stressed RBCs (sRBCs) place a large demand to quickly recycle these cells to prevent acute kidney injury and anemia. One mechanism employed in mice to accommodate this is the rapid recruitment of Ly-6C ^{High} monocytes derived from the spleen (Theurl et al. 2016). These monocytes, along with sRBCs, migrate to the liver where they differentiate into ferroportin expressing macrophages (Knutson et al. 2005). These transient macrophages are capable of effectively digesting sRBCs and recycling heme to be used in the production of new erythrocytes. This mechanism serves to increase production of inflammatory cytokines as well as myelopoiesis, resulting in the

upregulation of SPI-C and heme oxygenase-1 (HO-1), which are key factors in the SE pathway in mice (Theurl et al. 2016). HO catalyzes erythrophagocytosis in splenic macrophages, resulting in an increase of intracellular free heme as well as inducing heme-related. The inducible HO-1 isoform is upregulated during events of stress, serving as a good indication of stress induced damage of RBCs (Figure 2). Interestingly, the only known endogenous source of carbon monoxide (CO) is heme recycling, where HO catalyzes heme into biliverdin, releasing CO and iron into circulation. Therefore, increased circulating or exhaled CO levels may be used as biomarkers of increased RBC turnover rate (Tift et al. 2020).

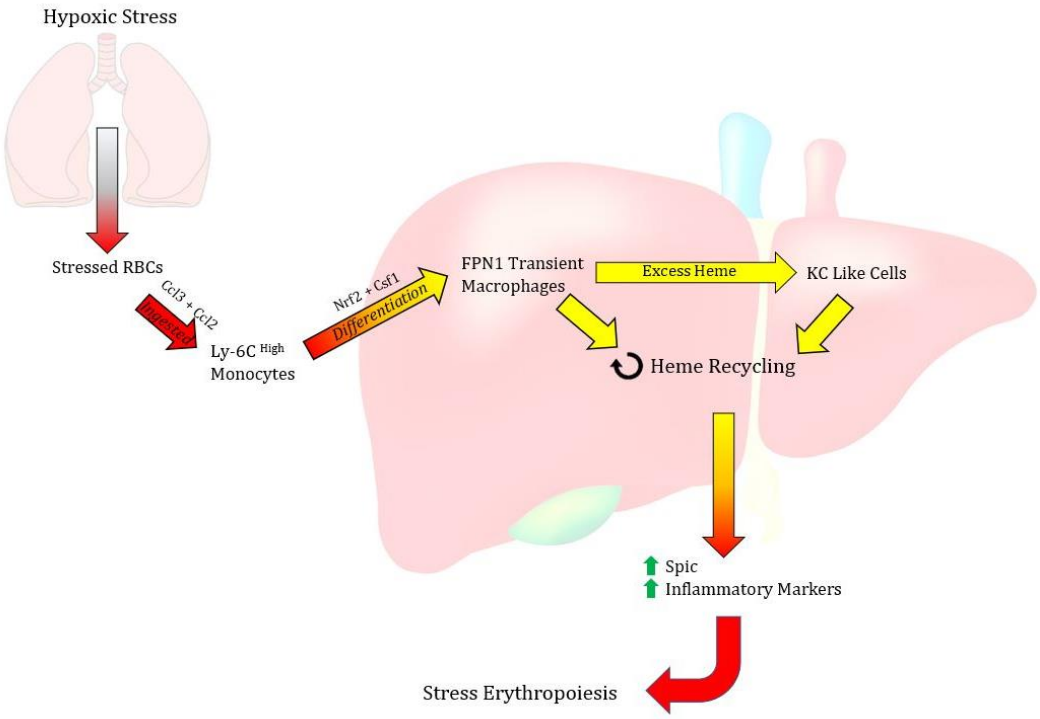

Figure 2. The mechanism of heme recycling during acute inflammation in mice. Inflammation induces the propagation of stressed erythrocytes (sRBCs) in circulation, resulting in large quantities of erythrocytes being degraded at once. Ly-6C ^{High} monocytes ($CD14^{High}CD14^{-}$ monocytes in humans) recruited from the spleen are released into circulation through the expression of chemokines Ccl2 and Ccl3. These monocytes, along with the sRBCs, are localized in the liver, where upon recognizing heme, Nrf2 and Csf1 expression increases, prompting the differentiation of Ly-6C monocytes into FPN-1 expressing transient macrophages. These transient macrophages are capable of digesting sRBCs and recycling heme to be used in erythropoiesis. In addition, markers of inflammation and hypoxia, such as Spic, are upregulated further contributing towards the initiation of the stress erythropoiesis mechanism. During events of high heme accumulation, transient macrophages may further differentiate into Kupfer like cells (KC Like cells) capable of digesting sRBCs more rapidly. As heme levels stabilize, these cells cease differentiation and the remaining cells remain arrested until they are digested themselves (Theurl et al. 2016).

Identifying RBCs Produced Through Stress Erythropoiesis

One promising marker of SE in humans is the increased concentration of fetal hemoglobin (HbF) in RBCs produced during hypoxic stress. HbF is an isoform of hemoglobin produced primarily in developing fetuses as a means to siphon oxygen from maternal blood. This occurs because HbF contains two gamma (γ) subunits in place of the two beta subunits normally seen in adult hemoglobin. This change confers a higher oxygen binding affinity, in part due to its inability to bind to 2,3 bisphosphoglyceric acid, a molecule that reduces oxygen affinity in hemoglobin. In adults, production of HbF is largely downregulated by the induction of the BCL11A complex(Sankaran et al. 2008; N. Liu et al. 2018), and 1-5% of all hemoglobin is HbF in normal healthy adults. However, pathological incidence of anemia and bone marrow transplants increase HbF concentrations between 4-10% 100 days after transplantation (Paulson et al. 2020). The exact mechanism of this inducible fetal erythropoiesis remains unclear; however, this discovery may provide insight into human SE. Similarly, one study showed an increase in fetal hemoglobin containing cells after 17 days of high-altitude exposure, in which individuals are exposed to chronic sustained hypobaric hypoxia (Paulson et al. 2020; Risso et al. 2012).

As described above, acute inflammatory stress coupled with hypoxia are sufficient to trigger a SE response in murine models. Therefore, if similar SE mechanisms occur in humans, then it is possible that new RBCs containing high HbF

levels which appear during acute anemia or inflammation are produced through the SE mechanism. Further support for this link is provided by the fact that erythroid progenitors from human thalassemia and sickle cell disease patients contain the same markers observed on murine and human BMP-4 dependent SE progenitor cells (CD34, KIT, and CD235a) and give rise to HbF-containing erythrocytes when cultured (Mathias et al. 2000; Luck et al. 2004). Since these severe acute inflammatory and hypoxic challenges likely induce the stress SE mechanism, it is predicted that RBCs produced via SE at high-altitude will also demonstrate elevated levels of HbF.

High-Altitude Hypoxemia

To determine if acute hypoxic exposure without secondary infection stimulates SE in humans, I will utilize a high-altitude exposure. Tissue hypoxia occurs when tissue metabolic oxygen demand exceeds supply and can result from a low oxygen supply in the blood (hypoxemia). Hypoxemia occurs at high-altitude due to decreased inspired oxygen partial pressures. Acute exposure to hypoxic conditions has been found to cause inflammation in the form of the upregulation of IL-6 as well as C Reactive Protein (CRP), a pro-inflammatory protein synthesized in the liver during acute inflammatory stress (Hartmann et al. 2000; Pham, Parikh, and Heinrich 2021). Similarly, intermittent tissue hypoxia, observed in patients suffering obstructive sleep apnea syndrome, results in the upregulation of the transcription factors Nuclear Factor κB (NF-κB) and Hypoxia Inducible Factor 1 (HIF-1) (Ryan, Taylor, and McNicholas 2005). These transcription

factors are key drivers of hypoxia-induced inflammatory cytokine production (T. Liu et al. 2017). Since acute high-altitude exposure induces both hypoxemia and concomitant systemic inflammation, I predict that SE is a key physiological response involved in high-altitude acclimatization.

Furthermore, in Andean high-altitude residents, maladaptive excessive erythrocytosis (EE) occurs in 33% of men by age 60 (Risso et al. 2012). EE is a primary contributor to development of Chronic Mountain Sickness (CMS), a progressive fatal disease associated with several symptoms and downstream comorbidities including sleep disturbance, reduced cognitive function, pulmonary hypertension, and stroke (Hancco et al. 2020). These symptoms are driven by severe hypoxemia and higher blood viscosity due to extremely high hematocrit levels (Villafuerte and Corante 2016). Previous evidence shows that individuals with EE have reduced arterial oxygen saturation, have limited peak cardiac output, show evidence of systemic inflammation, and experience increased bouts of sleep disordered breathing which result in continuous intermittent hypoxia during the night (Heinrich et al. 2020a). While the mechanisms responsible for EE are unknown, it has been shown that men with EE have higher instances of obstructive sleep apnea events (Heinrich et al. 2020b). It is possible that reoccurring nocturnal desaturation events and existing elevated inflammatory marker expression in this group could produce regular bouts of SE and contribute to the high hematocrit phenotype in this group.

It is possible that the acute hypoxemia and hypoxia-induced inflammatory response to high-altitude exposure induces SE in response to protect against tissue

hypoxia until the SSE mechanism recovers over several days of acclimatization. Furthermore, chronic stimulation of the SE pathway due to reoccurring severe desaturation events in long-term high-altitude residence may contribute to maladaptive polycythemia observed in some high-altitude resident groups. My research will provide key insight into whether SE is activated in humans in vivo at high-altitude. This would implicate SE as a key step in the process of acclimatization to high-altitude.

To determine if SE is occurring in individuals exposed acutely to high-altitude, I will measure biomarkers of burst formation of erythrocytes via the unique extramedullary SE mechanism in individuals at sea-level and throughout 3 days of acclimatization to 3800 m elevation (Hupperets et al. 2004). This analysis will include markers of inflammation as well as the quantity and expression of HbF in circulation. I hypothesize that if extramedullary SE is activated in humans at high-altitude then I will measure elevated levels of candidate biomarkers associated with SE in individuals exposed to 3 days at high-altitude. These candidate biomarkers of SE include: HbF, HO-1, and carbon monoxide (CO).

METHODS

Participant Recruitment

15 participants were recruited via word of mouth and flyers posted around the UCR campus. Participants were required to be over the age of 18 and fluent in English. Participants were excluded if they had a history of pulmonary or cardiovascular disease, were currently using anti-inflammatory medicine or other agents that may have interfered with ventilatory acclimatization to high-altitude (Basaran et al. 2016), were currently or recently treated for a systemic or serious local infection, traveled above 8,000 ft up to one month prior to the first measurements, were current smokers (cigarettes, e-cigarettes, or marijuana once a day), or were pregnant. Participant vital physiological measures were recorded at sea-level and are provided in Table 1. This study was approved by the UCR Clinical IRB and conducted in accordance with the *Declaration of Helsinki*, except for registration in a database. Participants were informed of the risks and benefits of the study and gave written informed consent to participate.

Table 1. White Mountain Study Participant Height, Weight, and Vital Measurements

	Men (n = 10)	Women (n = 5)	Overall (n = 15)
Age (Years)			
Mean ± SD	24.9 ± 4.28	26.4 ± 5.13	25.4 ± 4.45
Median [Min, Max]	23.0 [20.0, 32.0]	27 [19.0, 32.0]	24.0 [19.0, 32.0]
Weight (kg)			
Mean ± SD	80.7 ± 16.9	75.6 ± 22.3	79.0 ± 18.2
Median [Min, Max]	78.6 [54.0, 118]	68.0 [57.2, 114]	73.6 [54.0, 118]
Height (cm)			
Mean ± SD	175 ± 8.13	163 ± 9.42	171 ± 9.95
Median [Min, Max]	179 [157, 182]	160 [151,175]	171 [151, 182]
SBP (mmHg)			
Mean ± SD	130 ± 6.82	126 ± 8.87	129 ±7.43
Median [Min, Max]	132 [121, 138]	125 [118, 141]	126 [118, 141]
DBP (mmHg)			
Mean ± SD	79.4 ± 11.2	77.4 ± 8.35	78.7 ± 10.1
Median [Min, Max]	78.5 [63.0, 100]	79 [68.0, 88.0]	79.0 [63.0, 100]
BMI (kg/m^2)			
Mean ± SD	26.5 ± 5.40	28.2 ± 6.87	27.1 ± 5.74
Median [Min, Max]	24.8 [19.1, 36.8]	26.5 [22.3, 39.6]	25.4 [19.1, 39.6]

Blood pressure (BP) provided was averaged from four measurements taken on the first morning of the experiment at SL. SBP corresponds to Systolic Blood Pressure, while DBP corresponds to Diastolic Blood Pressure.

Study Design and Sample Collection

Participants arrived to the laboratory at UCR (~350 m elevation) at 7 am on the first day of the study. After completion of consenting procedures, several baseline (SL) physiological measures and blood samples were collected. Participants completed a medical history questionnaire which asked questions about their history of cardiopulmonary disease, smoking and drinking habits, ancestral background, and current medications. Acute Mountain Sickness (AMS) scores were collected using the Lake

Louise Scoring criteria, with research staff asking each question (Roach et al. 2018). Peripheral venous blood samples were collected during fasting by a licensed phlebotomist via standard venipuncture procedures via the medial cubital vein. 20 mL of blood was collected in EDTA vacutainer tubes (ThermoFischer Scientific Inc. Waltham, Massachusetts, USA) for proteomic analysis and ELISA, and 2.5 mL was collected in PaxGene Blood RNA tubes (ThermoFischer Scientific Inc. Waltham, Massachusetts, USA) to stabilize intracellular RNA for sequencing. Blood pressure was recorded with a Littmann Classic III Stethoscope & Prestige Sphygmomanometer (3M, Saint Paul, Minnesota, USA) after 5 minutes of resting in an upright seated position. Resting heart rate and SpO_2 were recorded with a Medtronic Nellcor N600 (Medtronic, Minneapolis, Minnesota, USA) and finger probe at rest in an upright seated position while breathing normally for 5 minutes. Exhaled carbon monoxide levels were recorded using a coVita Smokylyzer (coVita Inc., Santa Barbara, California, USA) following manufacturer instructions. Briefly, participants inhaled and held their breath for 10 seconds then exhaled complete through a tube into the device. Participants were provided with breakfast and refreshments following sample collection. Participants were then transported by car over a period of 9 hours to Barcroft Research Station in the White Mountain Research Center (3,800 m elevation).

Participants stayed at the station for 3 days, which is a sufficient timeframe to capture the majority of ventilatory acclimatization (Hupperets et al. 2004). Additional blood samples were collected each morning during fasting over the course of three days. Blood pressure, heart rate, pulse oxygen saturation, and AMS scores were recorded each

morning and evening. Exhaled carbon monoxide was recorded each evening as described previously. During the 3-day study, participants were provided 3 meals a day. One cup of coffee was permitted in the morning after measurements but before noon. Participants adhered to their typical sleep schedule but were required to wake by 9 AM to complete sample collection. To maintain consistent nomenclature, measurements or samples taken at sea-level will be denoted by SL, while any taken at high-altitude will be denoted by HA1, HA2, or HA3, with the particular day of the high-altitude study being represented by the respective number.

Blood Sample Processing

Immediately following collection, EDTA tubes were centrifuged at 2500 g for 10 minutes to separate plasma, buffy coat, and RBCs. Plasma was isolated, aliquoted, and frozen at -20 °C within 2 hours of collection. PaxGene Blood RNA tubes were stored at room temperature for 4 hours as per the manufacturer instructions, then frozen at -20 °C. Samples were transported to back to UCR in a liquid nitrogen dry shipper. Upon reaching the facilities at UCR, samples were stored at -80 °C until further processing. Due to lack of viability in some SL plasma samples, additional blood samples were taken as described above in September 2021 from the same subjects as well as new healthy participants matched to the original cohort by age, sex, and BMI. Statistical analysis showed no significant difference in BMI between matched participants within the two cohorts. ($t(10) = -0.319$, $p = 0.76$).

RNA Isolation

RNA from peripheral venous blood samples was isolated using the PAXGene Blood RNA Kit protocol, following the default procedure. The PaxGene system immediately stabilizes intracellular RNA in blood. Following isolation, RNA quality was preliminarily ascertained by measuring nucleic acid content via Nanodrop 2000 Spectrophotometer (ThermoFischer Scientific Inc. Waltham, Massachusetts, USA). Samples with A260/280 ratios of ~2.1 and A260/230 above 2.0 were utilized for sequencing. Samples were sent to the UC Riverside Genomics Core for further quality assurance using microcapillary electrophoretic RNA separation to determine RNA Identity Number (RIN) score (Schroeder et al. 2006). Samples with a RIN score of 8 or above were then prepared for RNA sequencing.

RNA-Sequencing

500 ng of input RNA of each sample were provided for library preparation with NEBNext Ultra ll Directional RNA Library Prep Kit (New England BioLabs, Ipswich, Massachusetts, USA). Standard manufacturers procedures were followed when preparing the samples, with the following exceptions: 0.8x beads were used during the first purification step after second strand synthesis, adaptor was diluted 1:15, 0.7x beads were used for purification after adaptor ligand, a total of 13 cycles of enrichment was done, and a dual bead size selection (0.5x and 0.7x) was used for size selection of adaptor ligated RNA. Library quality was verified via qPCR and samples were sent to UC San

Diego for sequencing using the Illumina NovaSeq 6000 (Illumina Inc., San Diego, California, USA).

RT qPCR

Real-time quantitative PCR (RT qPCR) was used to verify changes in *HBG1* and *HBG2* expression identified via RNA sequencing. I used the SYBR Green Quantitative RT-PCR kit (Sigma-Aldrich Inc., St. Louis, Missouri, USA) to quantify increases in fetal hemoglobin gene expression during high-altitude exposure. Isolated RNA samples taken from participants of the White Mountain study at SL and HA3 were for tested expression levels of *HBG1* and *HBG2* genes. These genes are responsible for encoding the γ_1 and γ_2 subunits, respectively, found in fetal hemoglobin.

Firstly, I designed unique forward and reverse primers to pinpoint a region in the third exon of each sequence to amplify each gene separately. Due to similarities in the coding regions of both *HBG1* and *HBG2*, a specific portion of the third exon was chosen as it contained the highest amount of variation between both genes and unique primer sequences targeting only one of the two genes could be obtained. The primers used to target these genes were designed using IDT RealTime qPCR Assay Designer:

HBG1 FWD: ATGTGCTGGTGACCGTTT

HBG1 REV: ATGTGTGATCTCTCAGCAGAATAG

HBG2 FWD: ATGTGCTGGTGACCGTTT

HBG2 REV: GTATCTCTTAGCAGAATAG.

Additionally, common housekeeping genes found in RBCs, *GAPDH* and *HPRT1*, would be included in the protocol to normalize *HGB1* and *HBG2* expression for statistical analysis. Both reverse and forward primers for these housekeeping genes were acquired from the ReadyMade Primer inventory provided by IDT (Integrated DNA Technologies Inc., Coralville, Iowa, USA).

GAPDH FWD: ACCACAGTCCATGCCATCAC

GAPDH REV: TCCACCACCCTGTTGCTGTA

HPRT1 FWD: CATTATGCTGAGGATTTGGAAAGG

HPRT1 REV: CTTGAGCACACAGAGGGCTACA.

Standard manufacturer protocols, detailed in the SYBR Green Quantitative RT-PCR kit, were followed for real-time PCR amplification of our target sequences. Due to a limited amount of space on the standard 96 well plate, two runs were conducted to include all available samples. The first run included samples taken from 4 participants, measuring *HBG1* and *HBG2* expressions between SL and HA3 samples, while the second round included samples taken from 5 different participants to avoid batch effect across paired samples. Both experiments were run identically, with the only difference being the removal of *HPRT1* in the second run as I determined the inclusion of *GAPDH* would be sufficient in the statistical analysis of the product. All PCR experiments were conducted with a QuantStudio 3 (ThermoFischer Scientific Inc. Waltham, Massachusetts, USA).

Preliminary visualization of qPCR output was observed using QuantStudio Design and Analysis Software v.1.5.1 (ThermoFischer Scientific Inc. Waltham, Massachusetts, USA).

Proteomic Analysis

Plasma samples obtained from 6 participants (3 males, 3 females) on SL, HA1, and HA3 were utilized for proteomic analysis. All samples were depleted using the Pierce Top 12 Abundant Depletion Spin Columns (ThermoFischer Scientific Inc. Waltham, Massachusetts, USA) following standard protocol. Depleted samples were frozen at -80°C and transported to our collaborators at UC San Diego on dry ice where our samples were received and processed. The samples were quantified using Label Free Quantification using Maxquant LFQ Algorithm run in a solution digest of LC-MS2 for 120 minutes using the Waters UPLC Hydrogen Deuterium Exchange Mass Spectrometer (Waters Corp, Milford, Massachusetts, USA) (Cox et al. 2014). Raw proteomic output from this protocol was analyzed and visualized in Peaks 8.5 developed by Bioinformatics Solutions (Waterloo, Ontario, Canada). Prior to protein quantification, plasma protein concentrations after depletion were validated by Bradford Assay using the Coomassie Bradford Protein Assay Kit provided by Thermo Fisher, following the manufacture's protocol with absorbance calibrated to 595 nm.

ELISA

To further verify the change in HbF protein as a result of high-altitude hypoxia, plasma HbF was measured via ELISA. I used the Human HbF/ Fetal Hemoglobin ELISA Kit from Lifespan Biosciences (Seattle, WA, USA). All 6 plasma samples taken from the SL cohort were included in the assay alongside 10 HA1 and 9 HA3 samples (this includes 6 matched and 3-4 unmatched samples). Additionally, RBC lysate isolated from the sea-level cohort was also included in the assay to determine if plasma HbF levels were correlated with cellular levels. RBC lysates were created using three freeze-thaw cycles. All plasma samples diluted were to a 1:10 ratio, while red blood cell lysate was done using a 1:20 ratio per recommendation of the manufacturer. Standard manufacturer protocol was followed regarding sample preparation and assay procedure.

Statistical Analysis

All statistical analyses were performed in R Studio (R v.4.1.0; R Foundation) or Excel (Microsoft, Redmond, WA, USA). Data is presented as mean ± standard deviation throughout this report. Asterisks indicate significant differences from SL at $p<0.05$(*), $p<0.01$(**), and $p<0.001$(***) levels. P values less than 0.05 were considered significant and adjusted p values are reported and evaluated where appropriate as indicated in the results section.

To evaluate changes in physiological variables across study days, repeated measured ANOVA was used with post-hoc pairwise t-tests with Bonferroni corrections

used to identify changes from sea-level values. These tests were done using the *aov* and *pairwise.t.test* function in the *multcomp* package in R. To determine if CO was elevated on HA1 and HA2 compared to SL values, paired t-tests were used, comparing each day at high-altitude to the SL value. Normality of data distributions was verified with Shapiro-Wilk tests prior to testing.

Ct values acquired from the output of both qPCR runs were used to determine changes in *HBG1* and *HBG2* expression across SL and HA3. Counts were normalized to *GAPDH* expression levels for delta-delta Ct method (Livak and Schmittgen 2001). Fold gene expression levels were log transformed prior to statistical testing. Differences in expression levels across days was determined using two-tailed paired t-tests in Excel.

Differential proteomic analysis was conducted on the output derived from the Maxquant PEAKS algorithm. This analysis was done in R using the *DEP* (v. 1.17.1) package (Zhang et al. 2018). The workflow outlined by Zhang et al. (2018) was followed when conducting the analysis, with the exception of the following modifications: Sex was added as an additional variable in the experimental design, a missingness threshold of 1 (thr = 1) was used for the data normalization and imputation step, and two samples were removed from the analysis (one from HA3 and SL respectively) due to large quantities of missing values that skewed data imputation.

RESULTS

Physiological Responses to High-Altitude

Physiological measures recorded at sea-level and over three days of acclimatization to high-altitude (3800 m elevation) are shown in Table 2. Resting heart rate significantly increased over the three-day period, with the baseline measurement of 78 ± 8.1 bpm at SL steadily increasing each morning at HA and peaking at 95.6 ± 12.8 bpm on HA3. Acclimatization can be seen in the average oxygen saturation (SpO_2) which drops from 94.8 ± 1.6 % at SL to 85 ± 4.4 % on HA1, 83.7 ± 2.5 % on HA2, then increasing to 86.1 ± 2.5 % on HA3. Similarly, AMS scores were highest on HA1 (3.1 ± 1.8), followed by a significant reduction over the subsequent two days to near baseline values (0.7 ± 1.2) on HA3, indicating successful acclimatization. There were no significant changes in systolic or diastolic blood pressure at rest at high-altitude. Average exhaled CO was found to be significantly increased on HA1 compared to SL ($t(28) = -2.57$, $p = 0.01$). A similar trend was observed when comparing SL to HA2, however this increase was not statistically significant ($t(28) = -1.91$, $p = 0.07$) (Figure 3).

Table 2. Physiological Measurements

Variable	SL	HA1	HA2	HA3	ANOVA
SBP	128 ± 7	125 ± 12	126 ± 45	126 ± 13	0.537
DBP	79 ± 10	83 ± 9	89.7 ± 12.1	85 ± 7	0.054
HR	78.0 ± 8.1	88.3 ± 13.2	89.7 ± 12.1*	95.6 ± 12.8 ***	<0.001
SpO_2	94.8 ± 1.6	85.0 ± 4.4***	83.7 ± 2.5***	86.1 ±2.5***	<0.001
AMS	0.2 ± 0.4	3.1 ± 1.8***	2.3 ± 2.0**	0.7 ± 1.2	<0.001

SBP - Systolic Blood Pressure (mmHg), DBP - Diastolic Blood Pressure (mmHg), HR - Heart Rate (bpm), SpO2 - Oxygen Saturation (%). Overall p-values for repeated measures ANOVA are provided. Asterisks indicate significant differences from SL at p<0.05(*), p<0.01(**), and p<0.001(***) levels via post-hoc pairwise comparisons with Bonferroni adjusted p-values.

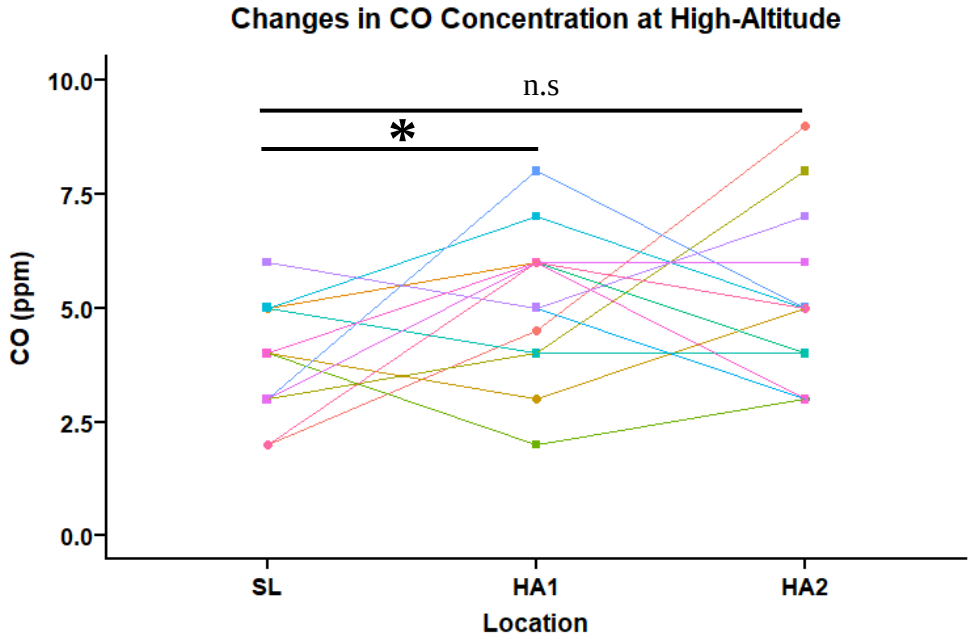

Figure 3. Changes in Exhaled CO Concentration During High-Altitude Acclimatization. A visualization of CO concentrations measured at SL, HA1, and HA2. A significant increase was seen between CO measured at SL and HA1 (p<0.05), while changes between SL and HA2 were not found to be significant (p = 0.06). Asterisks indicate significant differences from SL at p<0.05(*). Error bars represent 95% confidence intervals.

RNA-Sequencing

The expression of genes that code for candidate biomarkers of SE are found in Figure 4. Expression of *HBG1* and *HBG2* genes was found to be significantly increased on HA3 when compared to SL (adj. $p < 0.001$ for both genes). When compared to HA1, *HBG2* expression was significantly increased (adj. $p = 0.01$), while the increase noted in *HBG1* expression was not significant (adj. $p = 0.22$). Unexpectedly, expression of *HMOX1*, the gene that codes for HO-1, was found to be significantly decreased on both days at high-altitude (adj. $p = 0.003$ and adj. $p < 0.001$ respectively).

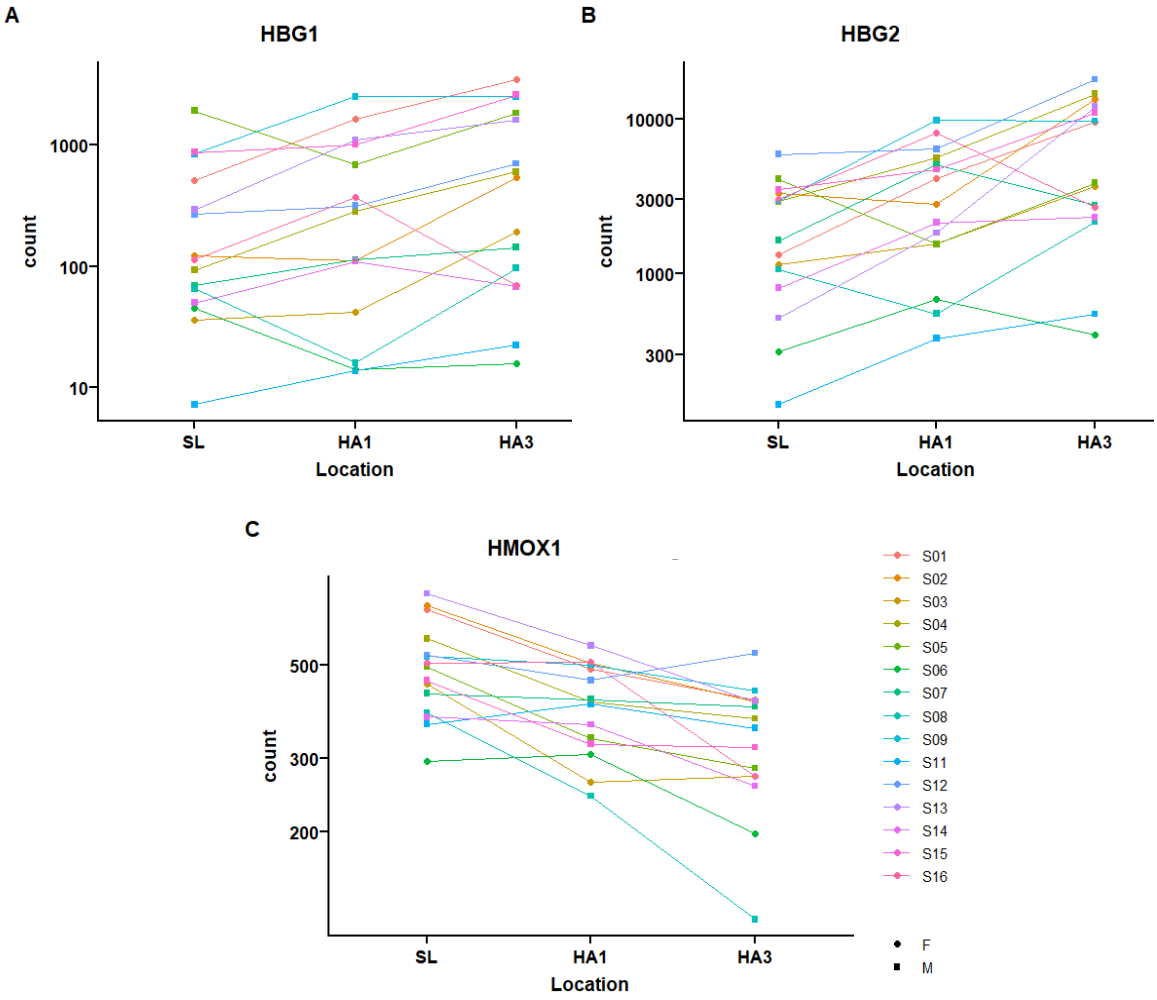

Figure 4. **Differential Gene Expression of Candidate Biomarkers of Stress Erythropoiesis.** RNA-Sequencing output detailing the transcription of a given gene separated by location of plasma sample collection. Fold change is represented as log10 normalized counts between SL, HA1 and, HA3. Results shown in **A** compare expressivity of the *HBG1* gene in participants at SL versus the HA1 AND HA3 respectively. Results shown in **B** and **C** illustrate the same comparison for *HBG2* and *HMOX1* respectively. Participants are represented in the figure through their identifying participant IDs as well as their biological sex.

RT qPCR

To validate the results from RNA sequencing, I conducted a qPCR analysis targeting the *HBG1* and *HBG2* genes, shown in Figure 5. Fold gene expression of both *HBG1* and *HBG2* increased in response to high-altitude acclimatization three days into the study, however this increase was only statistically significant in *HBG1* ($t(14) = 2.48$, $p = 0.03$), *HBG2* ($t(14) = 1.34$, $p = 0.20$). Interestingly, I noticed a sharp increase in the expression of *HBG1* in two participants within the high-altitude cohort. Due to the presence of outliers and low sample size ($n = 8$), I also conducted a Shapiro-Wilk analysis to check for normality in the data. Results from both *HBG1* and *HBG2* were found not to be normally distributed. Knowing this, I used the nonparametric Wilcoxon-Signed test of significance to verify my results. *HBG1* was found to be significantly increased ($p = 0.05$, $W = 9$), while *HBG2* was not significantly increased ($p = 0.46$, $W = 18$). This discrepancy may be a result of the outliers skewing the mean as well as the assumption of normality necessary for a T-Test.

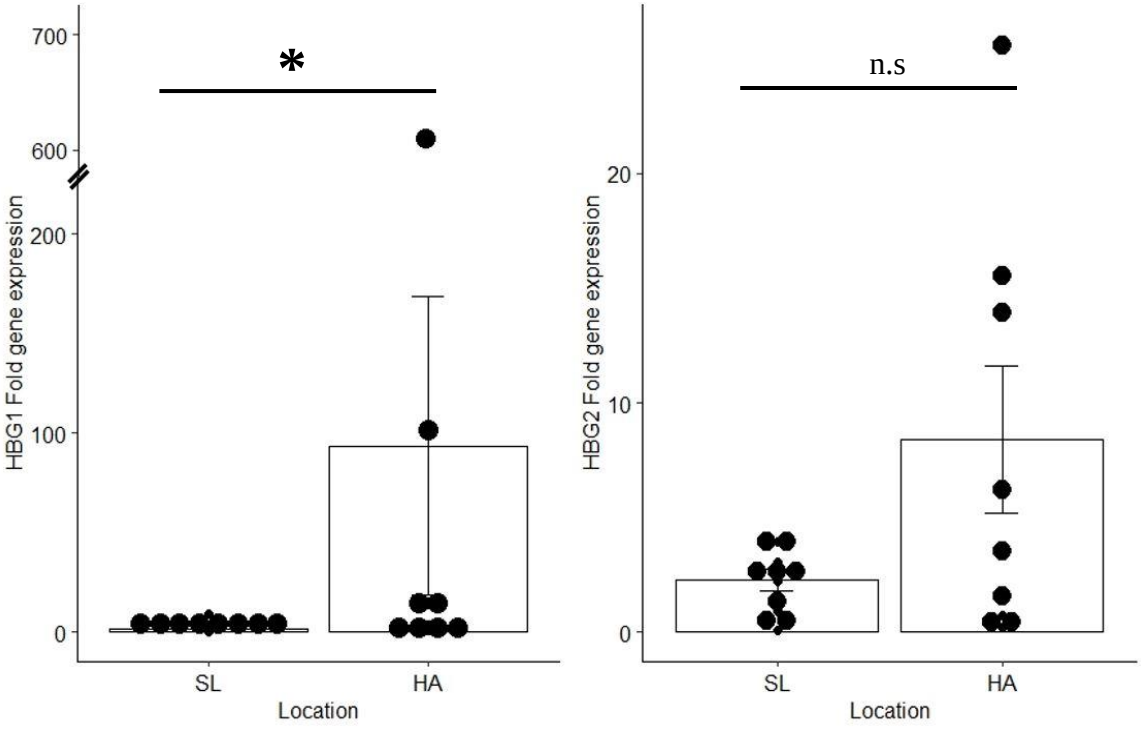

Figure 5. *HBG1* and *HBG2* Gene Expression Measured via qPCR. Fold gene expression of *HBG1* and *HBG2* comparing the RNA isolated on SL versus RNA isolated on HA3. Corresponding samples from 8 out of 9 participants from both qPCR runs are represented in this figure. Readings from one of the samples quantified in the second run returned inconclusive results, and as such were omitted. Asterisks indicate significant differences between SL and HA3 at $p<0.05$ (*). Error bars represent 95% confidence intervals.

Proteomic Analysis

Differential analysis of proteomic output was analyzed and visualized using the *DEP* workflow in R. During the analysis, it was revealed that imputation of missing values had a significant impact on the final results. This is a noted limitation of proteomic analyses due to the inevitability of missing values based on algorithm decisions during sample testing. Therefore, to account for varying significance levels as a result of

imputation of missing values, 10 identical runs using the *DEP* workflow were conducted. Proteins that were found to be significant in at least 8 out of 10 runs ($p < 0.05$) were deemed to be significant and were included in further validation. Results of a single run using the DEP workflow are presented in figures 6-8.

Additionally, select proteins that were found to be significantly and differentially expressed in this analysis were also observed during RNA sequencing (Table 3). Most notably, genes that coded for the proteins UBR4 and AXIN1 were found to be significantly decreased on HA1 and HA3 ($p <0.001$ for both days and genes), while the gene responsible for coding SBP2L was significantly upregulated on both days ($p <0.001$ for both days). Expression of the gene that codes AKAP1 was seen to be significantly downregulated but only on HA1 ($p = 0.03$). SBP2L and UBR4 are known to be crucial in regulating dietary selenium and maintaining cell membrane integrity respectively, whereas AKAP1 confers mitochondrial antiviral innate immunity localized in the outer mitochondrial membrane (Donovan and Copeland 2012; Yoshinaka et al. 2019; Rinschen et al. 2016). AXIN1 is a protein closely associated with the negative regulation of the Wnt signaling pathway and has been seen to possess tumor suppressing capabilities (Gordon and Nusse 2006; Satoh et al. 2000). Involved pathways identified using Gene Ontology of significantly expressed proteins can be found in Table 4.

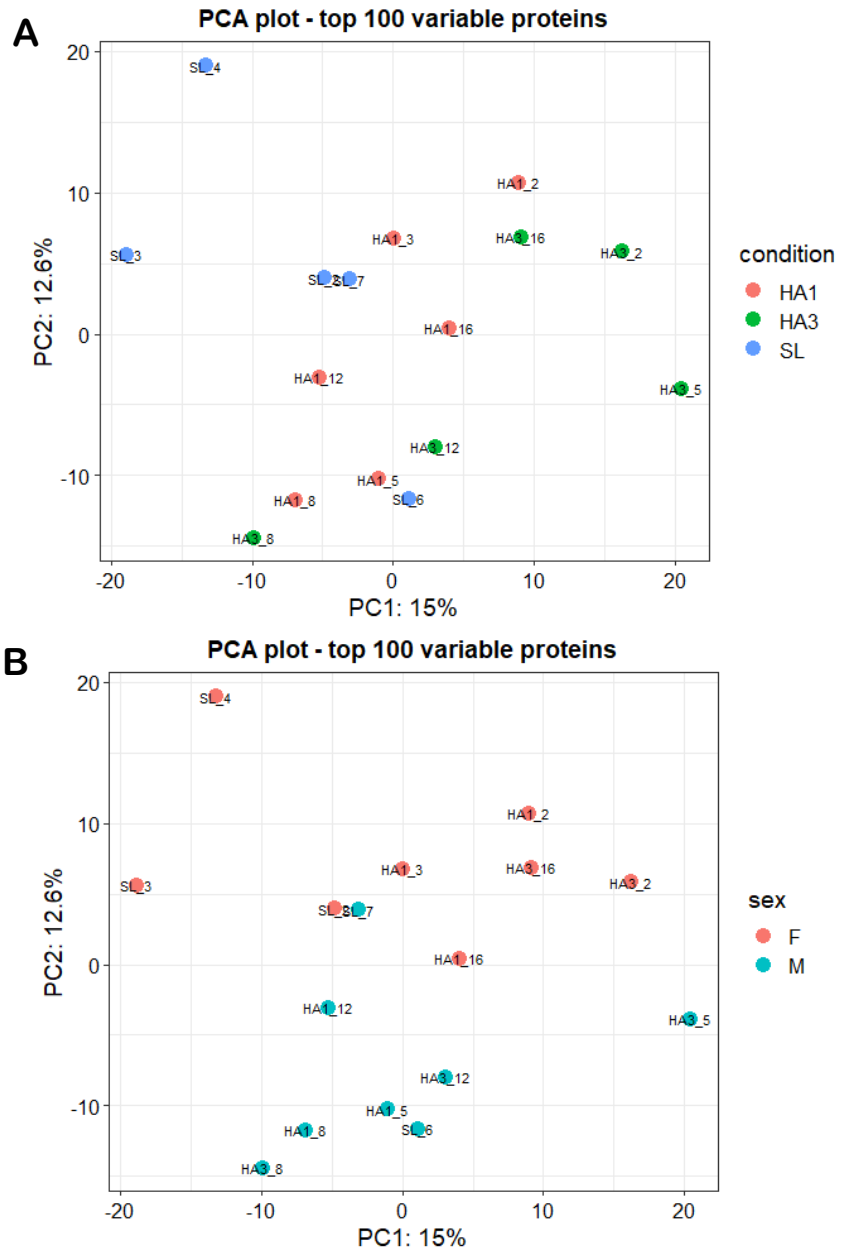

Figure 6. PCA Plots for Proteomic Analysis. Variations and clustering between samples condition and sex are denoted in **A** and **B**, respectively.

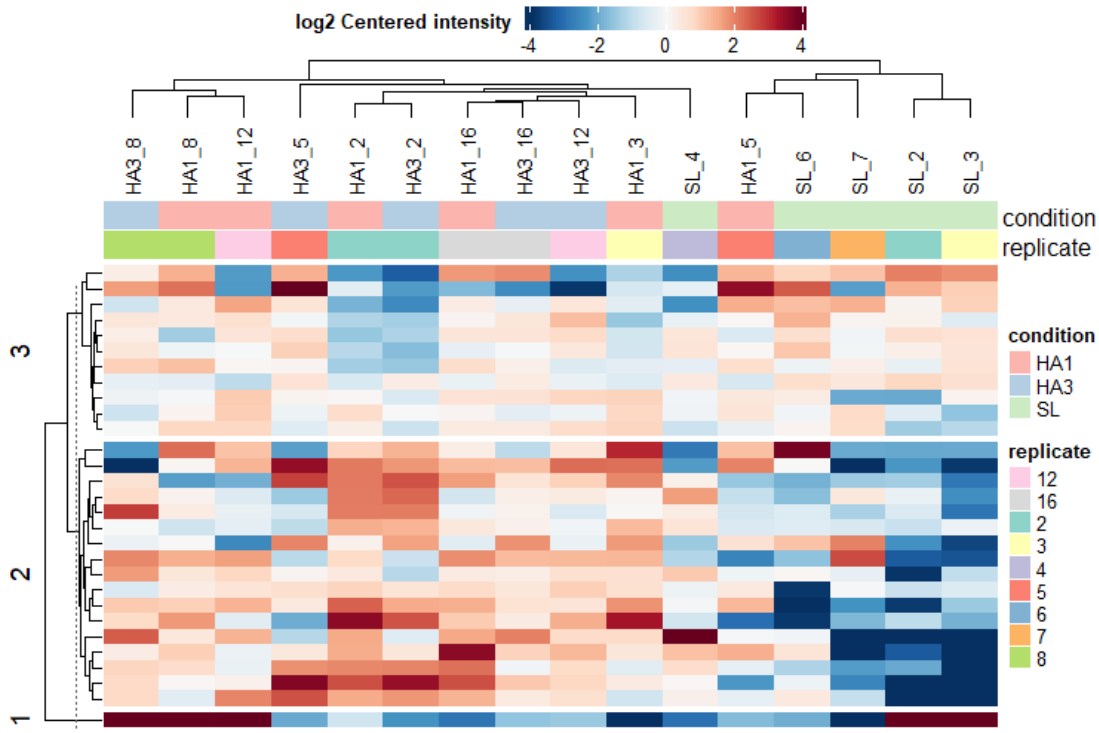

Figure 7. Heat Map of Top Differentially Expressed Proteins. Differential expression of significant proteins on a log2 centered scale when compared to each replicate (sample) and condition.

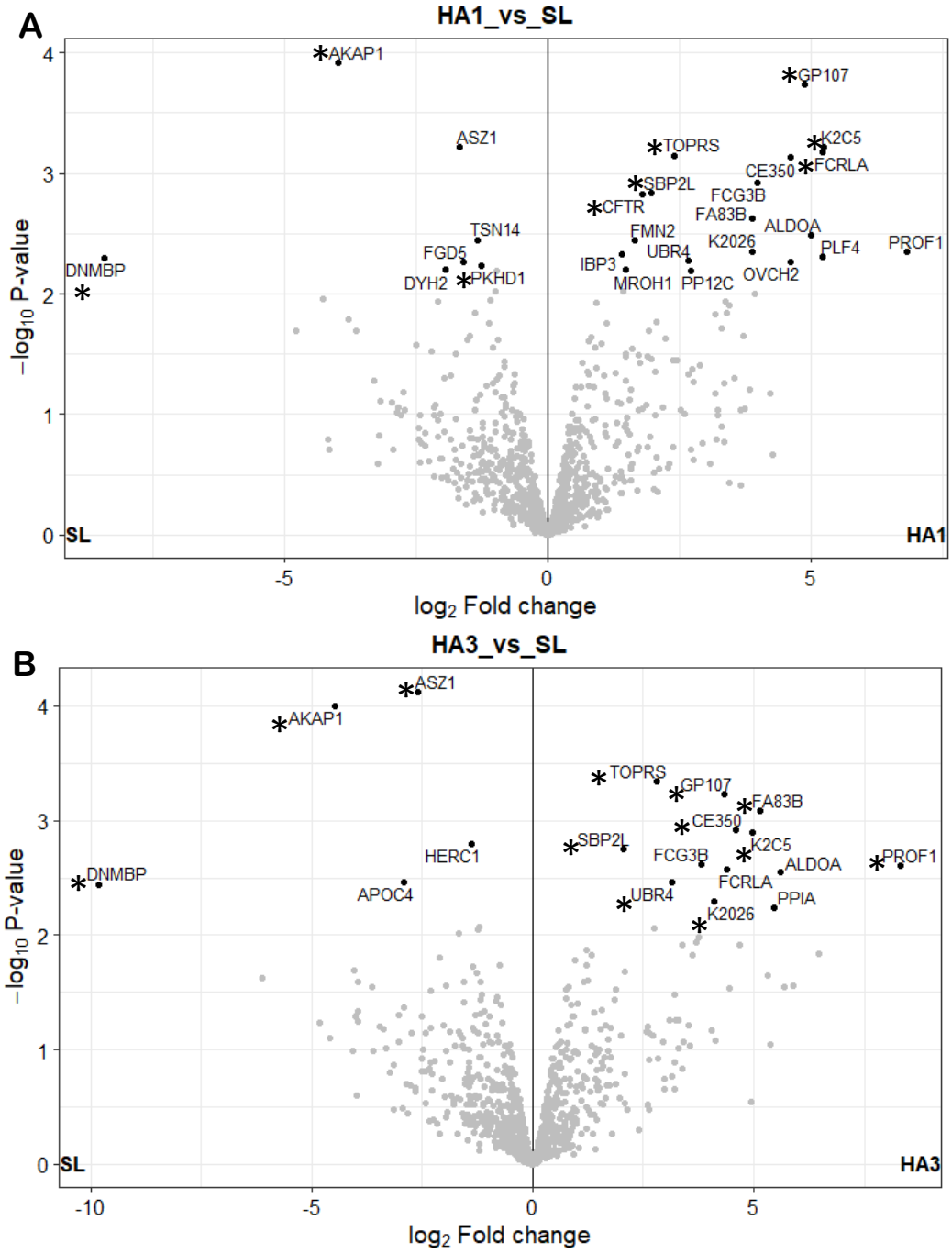

Figure 8. Volcano Plot of Protein Expression. A and **B** illustrate log2fold changes against their adjusted p-values for HA1 vs SL and HA3 vs SL respectively. Proteins labeled with an asterisk (*) were found to be significant in at least 8 out of 10 duplicate runs of this analysis.

Table 3: Differential Gene Expression of Significant Proteins

Gene Name	HA1vSL (p-value, log2foldchange)	HA3vSL (p-value, log2foldchange)
UBR4* **	<0.001, -0.529	<0.001, -0.408
SBP2L* **	<0.001, 0.507	<0.001, 0.471
AXIN1* **	<0.001, -0.516	<0.001, -0.558
AKAP1*	0.03, -0.226	0.54, -0.077
FCRLA	0.09, 0.212	0.69, 0.062
HERC1	0.16, -0.173	0.25, -0.149
DNMBP	0.59, 0.091	0.95, -0.013
CFTR	0.68, 1.452	0.81, 1.333
ASZ1	0.73, -1.299	0.85, -0.685
FMN2	0.83, 0.545	0.66, 1.122
OVCH2	0.98, 0.06	0.34, 2.106
PKHD1	0.99, -0.013	0.19, 1.415

Asterisks indicate a significant differential expression of a gene at (*) HA1 only, (**) HA3 only, or both HA1 and HA3 (***).

Table 4: Gene Ontology of Differentially Expressed Proteins

HA1 vs SL

GO Pathway	GO ID	p-value
Intracellular pH elevation	0051454	<0.01
Centrosome localization	0051660	<0.01
Actin bundle assembly	0030046	<0.01
Cellular response to forskolin	1904321	<0.01
Response to forskolin	1904322	<0.01
Regulation of epithelial cell apoptosis	1904035	0.01
Antiviral innate immune response	0140374	0.02

HA3 vs SL

GO Pathway	GO ID	p-value
Beta-catenin destruction complex assembly	1904885	<0.01
Antiviral innate immune response	0140374	0.01
piRNA metabolic process	0034587	0.01
Beta-catenin destruction complex disassembly	1904886	0.01
Peptidyl threonine phosphorylation	0010800	0.02
Ubiquitin protein transferase activity	0051438	0.02
Proteolysis of cellular protein catabolic processes	1903052	0.02

ELISA

Analysis of HbF concentrations referencing the acquired absorbance vs optical density indicates proper binding of HbF to both plasma and cell lysate. I found that both cell lysate and plasma samples taken from the same cohort correlate well ($R^2 = 0.75$). Statistical analysis of SL samples compared to that of HA 1 did not result in a significant difference between cohorts ($t(14) = 0.66$, $p = 0.52$). This trend held true when comparing SL to HA3 ($t(15) = 0.56$, $p = 0.54$) (Figure 9).

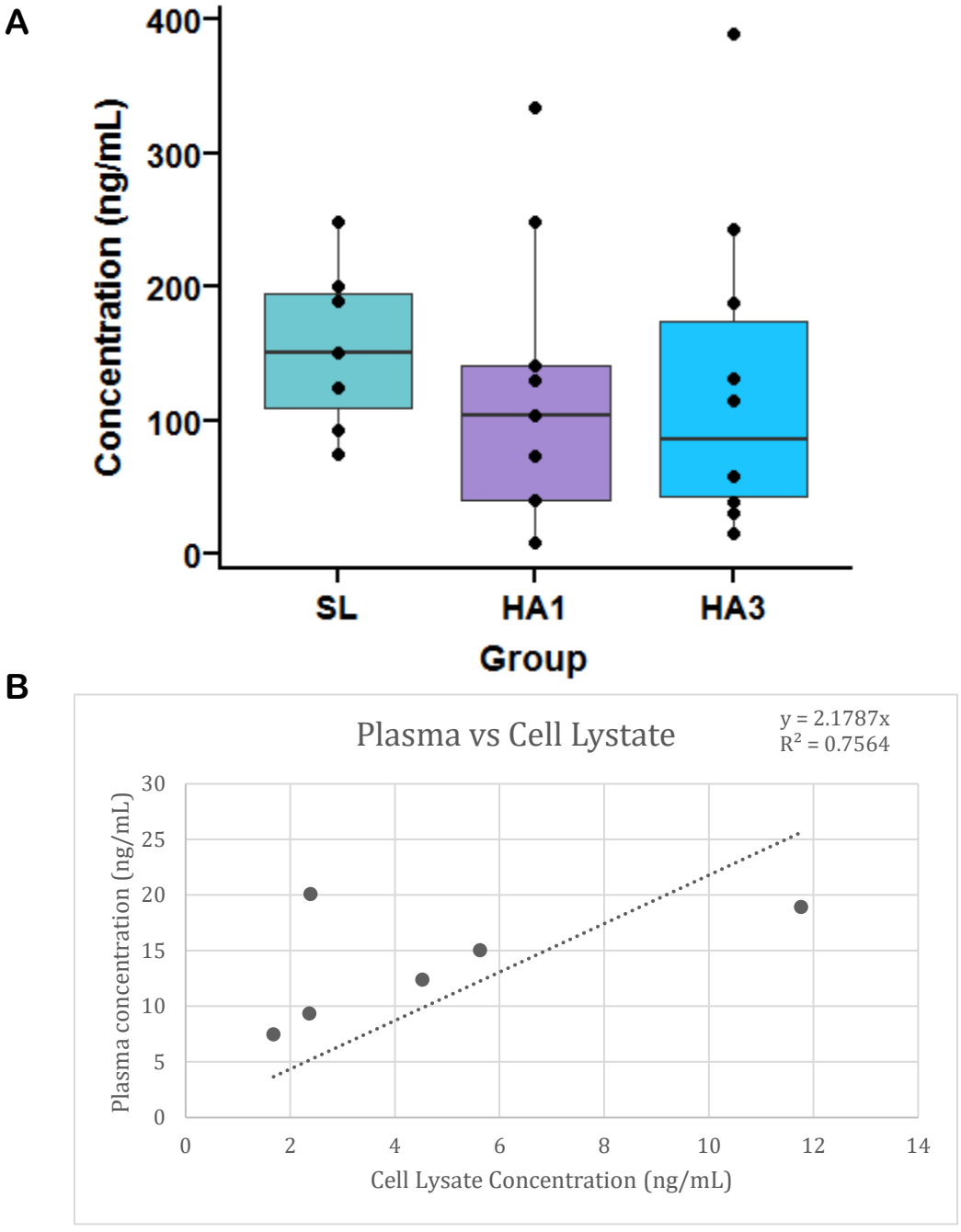

Figure 9. HbF Enzyme-Linked Immunoassay of Plasma and Cell Lysate. A. Concentrations of HbF estimated from collected plasma samples taken at SL (n = 7), HA1 (n = 10), and HA3 (n = 9). **B.** Correlation plot comparing concentrations of plasma and cell lysate samples taken from the same participant (n = 6).

DISCUSSION

Biomarkers of High-Altitude Stress Erythropoiesis

This study investigates the prevalence of HbF, CO, and HO-1 as biomarkers of high-altitude SE. Firstly, I quantified evidence of the upregulation of HbF at a proteomic level using an ELISA as well as at the transcriptomic level through RNA sequencing and quantitative PCR. These findings correlate well with similar studies observing HbF in unacclimatized individuals exposed to high-altitude for longer time periods (17 days) (Risso et al. 2012). Similarly, my analysis of participants at HA1 revealed a significant increase in CO when compared to SL (Figure 3). Interestingly, elevated CO levels have also been observed in lifelong high-altitude Andean residents compared to sea-level residents and carboxyhemoglobin levels are positively associated with hematocrit in this group (Tift et al. 2020). This provides further evidence that CO levels are linked to RBC turnover rate and are elevated at high-altitude in both sojourner and resident populations.

Interestingly, RNA sequencing revealed downregulation of *HMOX1*, the gene responsible for coding HO-1 (Figure 4). One possible explanation for this may be the upregulation of myelopoiesis that occurs during inflammation. Myeloid progenitor cells, specifically neutrophils, which are recruited do not possess HO-1 and as such traces of *HMOX1* containing mRNA would not as abundant in the plasma. Moreover, the compartmentalization of SE would also skew the association between HO-1 abundance in the plasma compared to that of circulating plasma. Nonetheless, these conflicting results

may provide insights into the proposed adaptive HO-1/CO pathway during hypoxia (Tift et al. 2020).

Additional Insights from Proteomic Analyses

Intracellular pH Regulation as a Response to Hypercapnia by CFTR

Results from our differential analysis of plasma revealed a significant group of proteins responsible for maintaining metabolic homeostasis during bouts of inflammation. In particular, I observed the upregulation of the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-binding cassette transporter that facilitates the flow of Cl^- ions within respiratory epithelial cells (Hyde et al. 1990; Sheppard and Welsh 1999). CFTR activity is regulated by intracellular pH, such that alkaline intracellular pH inhibits the structural motif of the ATP binding subunits, whereas acidic intracellular conditions allow this cascade to proceed to completion (J.-H. Chen, Cai, and Sheppard 2009). Moreover, CFTR activity has also been seen to be influenced by a ventilatory response to hypercapnia, the presence of CO_2 circulation. This association was found in a mouse model in which *Cftr* knockout prevented increased ventilation in hypoxia (Bonora et al. 2004). It is important to note that Cystic Fibrosis is a genetic disorder that causes disfunction of the CFTR ion channel. However, this does not affect our findings, as no participants presented with this disease. Interestingly, I found that CFTR was significantly upregulated on HA1 but not on HA3. A possible explanation for this occurrence could be that participants on the first day were in the processes of

acclimatizing to the hypoxic environment. Moreover, the increase in CFTR activity may correlate to hypercapnia induced by hyperventilation. This interaction may serve as a metabolic adaption to prevent blood alkalization, a response typically during high-altitude acclimatization.

AXIN1 Regulation of Wnt Signaling

Wnt signaling is an evolutionarily conserved metabolic cascade responsible for determining cell differentiation, growth, and migration (Cadigan and Peifer 2009; van Amerongen and Nusse 2009). Without activation of this pathway, β-catenin, the primary transcription factor responsible for regulating cell fate, is phosphorylated and degraded by adhering to protein complex composed of AXIN, adenomatosis polyposis coli (APC), and a family of kinases and phosphatases (Gordon and Nusse 2006; Komiya and Habas 2008). The AXIN protein complex is inhibited in the presence of Wnt ligand, allowing the for the accumulation of β-catenin as well as its downstream effects on gene expression.

In the context of erythropoiesis, Wnt signaling serves an important role the SE pathway by activating the proliferation of stressed erythroid progenitor cells, while simultaneously inhibiting their differentiation (Y. Chen et al. 2020). Moreover, Wnt signaling plays a crucial role in vascularization, the generation of generation of new blood vessels as a means to increase blood flow throughout the body (Goodwin and D'Amore 2002; Olsen et al. 2017). While beneficial at a homeostatic level, unregulated activation of the Wnt signaling pathway can become pathogenic, such as the through the

growth of different forms of cancers (Zhan, Rindtorff, and Boutros 2017). For this reason, AXIN1's ability to inhibit the Wnt signaling pathway often categorizes it as a cancer suppressing agent.

My results indicate a significantly increased level of AXIN1, a member of the AXIN family of proteins, in participants on HA3, suggesting an inhibitory response to the Wnt signaling pathway. The implication of these results remains inconclusive, as protein samples were quantified using circulating plasma and might not be representative of the splenic niche detailed in this study. Nonetheless, this observation may provide insight into the role of the canonical Wnt signaling and its regulation in SE and adaptive physiology as a whole.

Analysis of Bioinformatics

Upon further investigation into the DEP workflow used for proteomic analysis, it was discovered that duplicate runs using this workflow would return different results. Specifically, following the manufacturer's protocol as well while holding run conditions constant resulted in a varying amount of significant differentially expressed proteins. This outcome was a consequence of the data imputation and normalization steps of the workflow, which estimate missing values in the data set for an accurate comparison.

For the scope of this study, I opted to conduct 10 identical runs of the DEP workflow as a means to elucidate proteins that would be consistently marked as significant ($p < 0.05$). All runs were held using the default workflow, with the only

modification being the removal of two plasma samples, one being taken from HA3 and SL respectively. This was done to reduce the number of missing values present in the dataset. The results of the 10 runs are found in figure 10. Proteins that were significantly expressed in at least 8 out of 10 runs were considered to be significant for the purposes of the above analysis.

This discrepancy has been the topic of a recent study which investigate the confidence and computation error of proteomic differential analysis (Jin et al. 2021). Moreover, future studies implementing proteomic differential analysis, as well as other tests of significance, should be mindful of the assumptions made through data estimation and how this may manifest in their data.

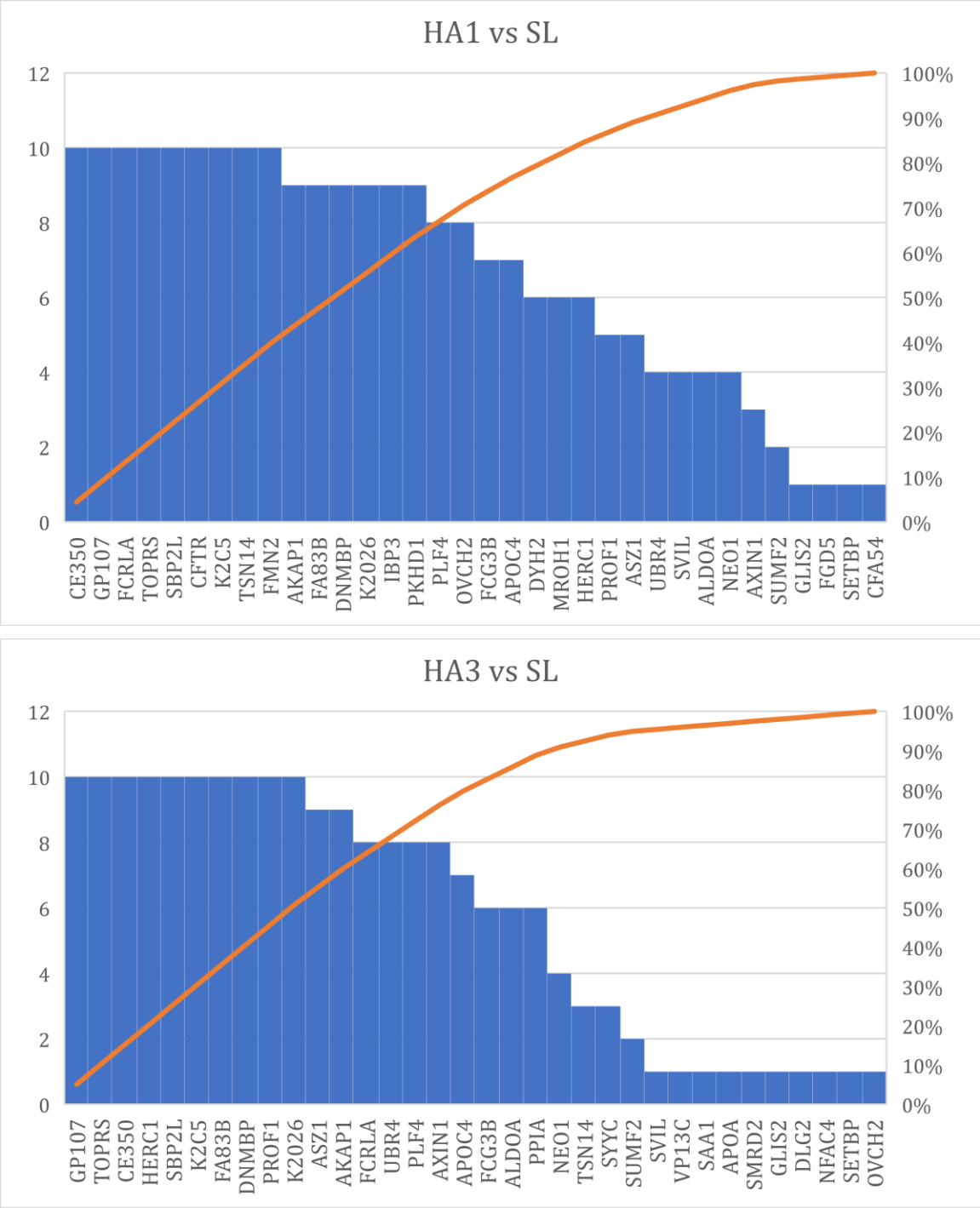

Figure 10. Significantly Expressed Proteins Identified in 10 Runs of the DEP Workflow. A figure showing the frequency of significantly expressed proteins observed in 10 replicate runs using the DEP workflow. Percentages represent the frequency at which proteins were marked as significant ($p < 0.05$) between 10 runs. **A** and **B** list all significant proteins identified at HA1 vs SL and HA3 vs SL respectively.

Limitations

One limitation of this study was the relatively small sample size used for proteomic differential analysis (n = 6). This is also confounded in part due to the mixed cohort used to compare SL to HA1 and HA3. Additionally, the prevalence of missing values in output from protein quantification resulted in the further removal of two samples (SL_5 and HA3_3) in order to minimize error as a result of data imputation (Figure 11). Moreover, samples from all experiments we derived from whole blood, and as such may not represent the particular splenic niche that is characteristic of SE. Nonetheless, these findings underscore effects of high-altitude hypoxia on the anemia of inflammation and erythropoiesis in humans.

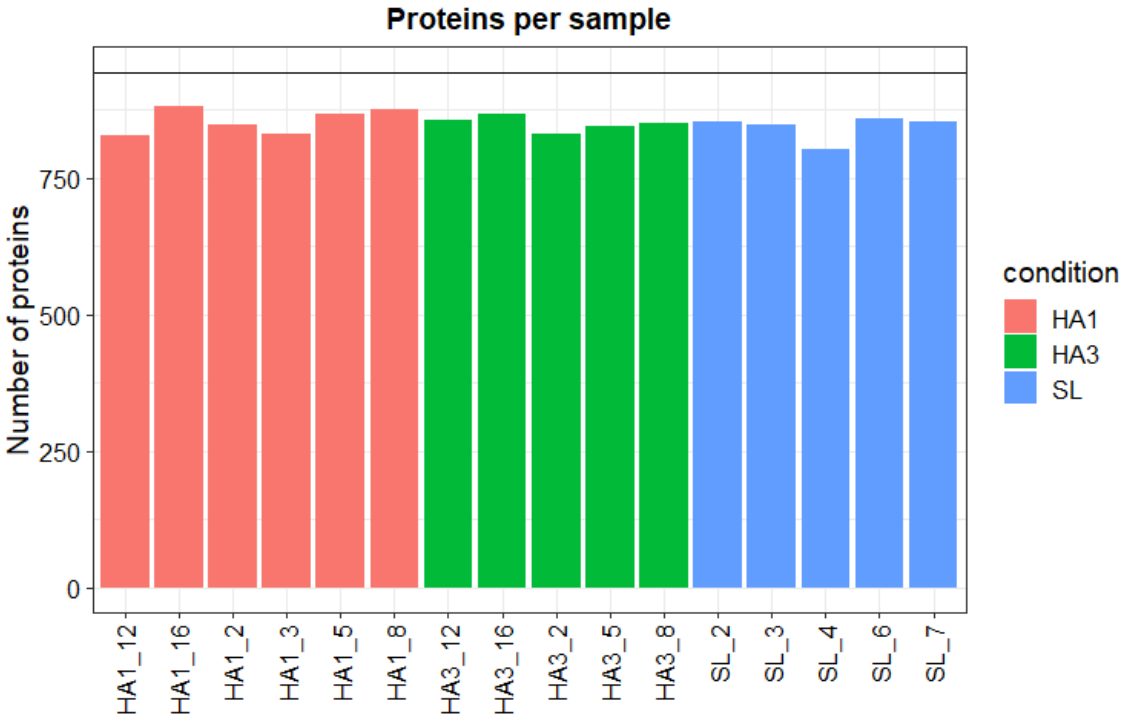

Figure 11. Protein Quantification and Imputation. Results taken from the DEP Proteomic Analysis workflow quantifying the number of unique proteins found in each sample after data normalization. Samples HA3_3 and SL_5 were removed from this analysis, as they contained a larger quantity of missing values compared to the remining samples. This was done to reduce the effects of data imputation on the determination of protein significance further along in the workflow.

Future Directions

The insights gleamed from this study will serve as a basis for further investigation into the role of high-altitude hypoxia in SE. Future studies observing unacclimatized individuals at high-altitude may choose to prolong the time spent in hypoxic conditions. Additionally, researchers ought to recruit a larger cohort of participants consisting of an equal number of men and women, as many of the correlations identified in this study were most strongly linked to biological sex. Moreover, research into populations residing at high-altitudes such as the aforementioned Andean community, may also reveal the inner workings of erythropoiesis when exposed to high-altitude hypoxia chronically. Such findings will prove invaluable when determining the long term physiological and pathological effects chronic hypoxemia in the context of SE and other adaptive physiological mechanisms

CONCLUSION

In this study, I identified key biomarkers of the stress-dependent erythropoiesis pathway in humans. I validated this claim by quantifying the upregulation of these biomarkers at a transcriptomic, proteomic and physiological level in individuals exposed to acute hypoxemic stress at high-altitude. Moreover, I examined additional biomarkers that interact with key pathways of SE, such as regulation of blood pH and Wnt signaling. It is clear that acute exposure to high-altitude hypoxia in unacclimatized individuals contributes to the presence of biomarkers of SE. However, it remains to be seen how chronic activation of SE may manifest in populations residing at high-altitude, such as the aforementioned Andean communities. Furthermore, these findings paint a clearer picture of SE in humans and its connection to systemic inflammation.

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