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Integrative approaches to understanding human adaptation and acclimatization to high altitude

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

James J. Yu

Committee in charge:

Professor Tatum S. Simonson, Chair
Professor Pascal Gagneux
Professor Amy L. Non
Professor Frank L. Powell

2024

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University of California San Diego

2024

DEDICATION

For my parents, who immigrated to the United States and gave up everything so that their children could have anything.

EPIGRAPH

In a letter from John Steinbeck to his friend and editor, Pascal Covici, on finishing his novel *East of Eden*:

> As you know, a prologue is written last but placed first to explain the book's shortcomings and to ask the reader to be kind. But a prologue is also a note of farewell from the writer to his book. For years the writer and his book have been together–friends or bitter enemies but very close as only love and fighting can accomplish.
>
> Then suddenly the book is done. It is a kind of death. This is the requiem.
>
> Miguel Cervantes invented the modern novel and with his Don Quijote set a mark high and bright. In his prologue, he said best what writers feel–The gladness and The terror.
>
> "Idling reader" Cervantes wrote, "you may believe me when I tell you that I should have liked this book, which is the child of my brain, to be The fairest. The sprightliest and The cleverest that could be imagined, but I have not been able to contravene the law of nature which would have it that like begets like–"
>
> And so it is with me, Pat. Although some times I have felt that I held fire in my hands and spread a page with shining–I have never lost the weight of clumsiness, of ignorance, of aching inability.

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ABSTRACT OF THE DISSERTATION

Integrative approaches to understanding human adaptation and acclimatization to high altitude

by

James J. Yu

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2024

Professor Tatum S. Simonson, Chair

High altitude and the hypobaric hypoxia associated with it impose significant physiological stressors on the human body. The human response to hypoxia varies between and within populations of differing genetic ancestries. This variation provides a natural experiment where potential mechanisms of hypoxia response can be investigated, especially for groups that have adapted to high altitude. This dissertation investigates the human response to hypoxia and high

altitude by studying distinct populations through various biological approaches. The first chapter combines genetics, proteomics, and metabolomics data from Andean highlanders into a multi-omics analysis to predict phenotypes related to high-altitude adaptation, such as basic physiological traits, hematological traits, metabolism, and ventilation. The analysis identifies key contributors from each of the different -omics to various phenotypes. The second chapter characterizes the hypoxic ventilatory response in Tibetan women residing at high altitude and identifies an association between menopausal status and ventilation. The study further identifies genetic associations with ventilatory traits and genes under positive selection that may play a role in control of breathing. The third chapter is the results of a randomized, double-blinded, placebo-controlled study testing the effect of the probiotic SLAB51 on sea-level residents acclimatizing to high altitude. The study shows individuals that ingest the probiotic have a higher oxygen saturation and less symptoms of Acute Mountain Sickness at high altitude than those that receive a placebo. Taken together, this dissertation provides novel insights into how humans adapt and acclimatize to high altitude.

INTRODUCTION

As modern humans migrated outside of the African continent in the last 100,000 years, they encountered various landscapes and environments (Nielsen et al., 2017). In order to survive and reproduce, humans adapted to their local environments through the process of evolution through natural selection (Fan, S. et al., 2016). Some of these environments lay at the extremes of what is considered habitable, ranging from arid, hot deserts to the cold, unforgiving arctic, yet in a span of approximately 50,000 years, humans had permanently occupied almost every landscape on Earth, reflecting the incredible ability of humans to adapt to their local environments (Nielsen et al., 2017). At the heart of this ability to adapt is the genome, which through random mutations can be acted upon to choose heritable traits that are advantageous for survival and reproduction. A chief example of this incredible adaptability are human populations that reside at high altitude.

High altitude (>2500 m) imposes significant stressors on the human body. The combination of cold temperatures, UV radiation exposure, and reduced available oxygen due to hypobaric hypoxia create an extreme environment, with the last of these traits creating the strongest physiological pressure. Yet, despite these harsh conditions, humans have permanently settled high-altitude regions on three different continents for hundreds of generations (Hassen, 1990; Ossendorf et al., 2019; Rademaker et al., 2014; Zhang, X. L. et al., 2018). These populations primarily include Tibetans on the Tibetan plateau, Andeans on the Andean altiplano, and Ethiopians on the Ethiopian plateau. Based on archeological evidence, these populations have occupied their respective highland regions for varying lengths of time (Fig. 0.1).

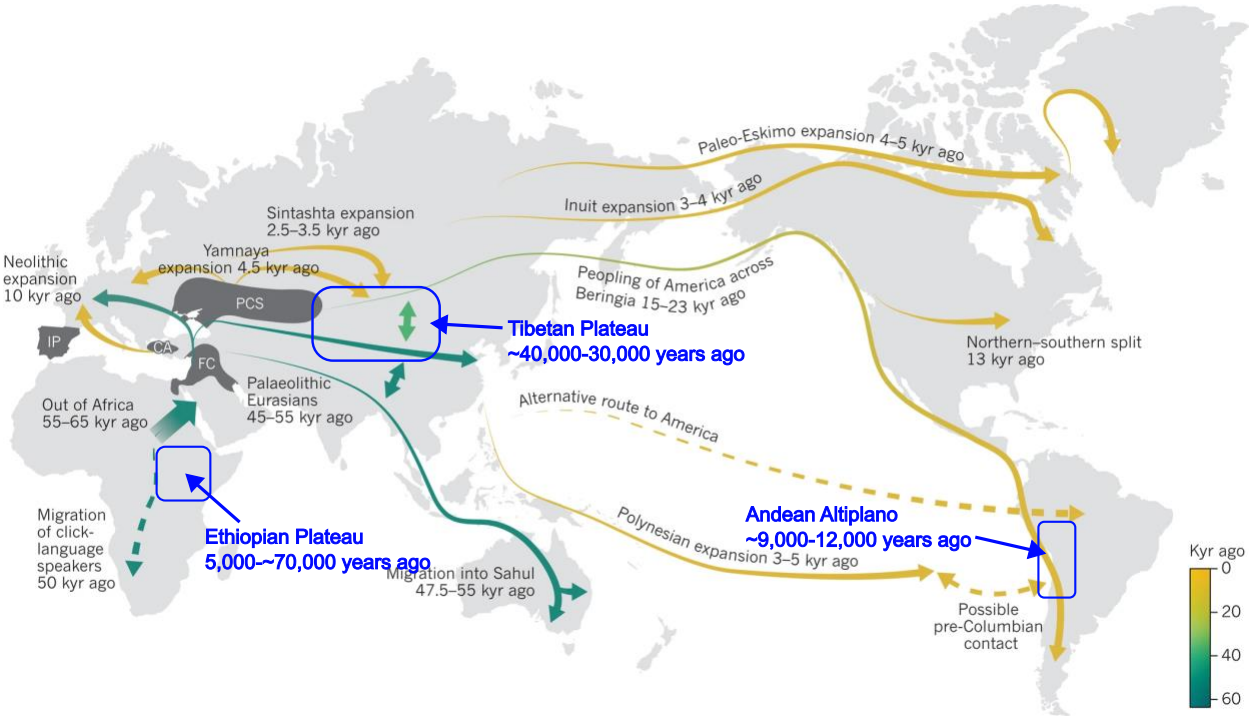


Figure 0.1: Figure adjusted from Nielsen et al. (2017) depicting human migration outside of Africa along with time ranges of when they occupied high altitude environments.

Despite the common physiological pressure of hypobaric hypoxia, these different high-altitude populations predominantly exhibit distinct physiological traits. Compared to lowlanders that move to high altitude, Tibetans generally have lower hemoglobin concentration ([Hb]), larger lung volumes, higher birth weights, higher resting ventilation, and higher hypoxic ventilatory responses (HVR) (Beall, 2007; Beall et al., 1997; Curran et al., 1997; Droma, T. et al., 1991; Hackett et al., 1980; Sun, S. F. et al., 1990; Zamudio, S. et al., 1993; Zhuang, J. et al., 1993). Andeans generally have higher [Hb], larger lung volumes, higher birth weights, and lower HVRs (Beall, 2007; Bigham et al., 2014; Brutsaert, T. D. et al., 2000; Brutsaert, T. D. et al., 1999; Heinrich et al., 2020; Julian et al., 2011; Julian et al., 2009). Ethiopians have been less studied than their Tibetan and Andean counterparts, but studies indicate they have lower [Hb] similar to

Tibetans (Beall et al., 2002) and higher oxygen saturation (SpO_2) than both Tibetans and Andeans (Beall, 2006). Despite these general patterns of adaptation for each group, there is also considerable amounts of variation within each population (Beall, 2007; Yu, J. J. et al., 2022), indicating that natural selection hasn't completely driven the respective populations to a fixed phenotype. Additionally, for some of these phenotypes, it is unclear if these general patterns are adaptive or simply physiological responses to chronic hypoxia exposure.

Many of these traits have a clear hereditary component, indicating their etiology and transfer to offspring are at least partially due to changes in the genome (Yu, J. J. et al., 2022). Genomes contain clues to their evolutionary history that can be pieced together to form evolutionary analyses (Akbari et al., 2018; Grossman, S. R. et al., 2010). Indeed, analyses such as positive selection scans reveal that these high altitude populations' genomes have undergone transformations in response to high altitude, including some of the fastest allele frequency changes ever observed in humans (Yi, X. et al., 2010). Additionally, many of these positive selection scans have replicated findings of genes within the hypoxia-inducible factor (HIF) pathway as primary targets of selection within Tibetans (Beall et al., 2010; Simonson et al., 2010; Xu, S. et al., 2011), Andeans (Bigham et al., 2010; Lawrence et al., 2024), and Ethiopians (Huerta-Sánchez et al., 2013; Scheinfeldt et al., 2012). As a master regulator of the body's response to hypoxia (Semenza, 2012; Semenza et al., 1991; Wang, G. L. et al., 1995), the HIF pathway remains a primary suspect of genetic adaptation in these highland populations.

Positive selection scans reveal both divergent and convergent results in what genes are under positive selection depending on the population (Fig. 0.2). A key player within the HIF pathway is the gene *endothelial PAS domain protein 1* (*EPAS1*), which encodes for the HIF2-α subunit. Identified as a target of natural selection in the earliest positive selection scans within

Tibetans (Beall et al., 2010; Bigham et al., 2010; Simonson et al., 2010), more recent studies have identified *EPAS1* as also being under positive selection within Andean highlanders, though the specific genetic mutations differ between Tibetans and Andeans (Eichstaedt et al., 2017; Jorgensen et al., 2023; Lawrence et al., 2024). A similar trend exists for *egl-9 family hypoxia inducible factor 1* (*EGLN1*), which encodes for HIF prolyl 4-hydroxylase 2 (PHD2); studies have identified *EGLN1* as a target of selection in both Tibetans and Andeans (Bigham et al., 2010; Bigham et al., 2013; Simonson et al., 2010; Xu, S. et al., 2011), but the actual genetic variants differ between the two populations (Heinrich et al., 2019).

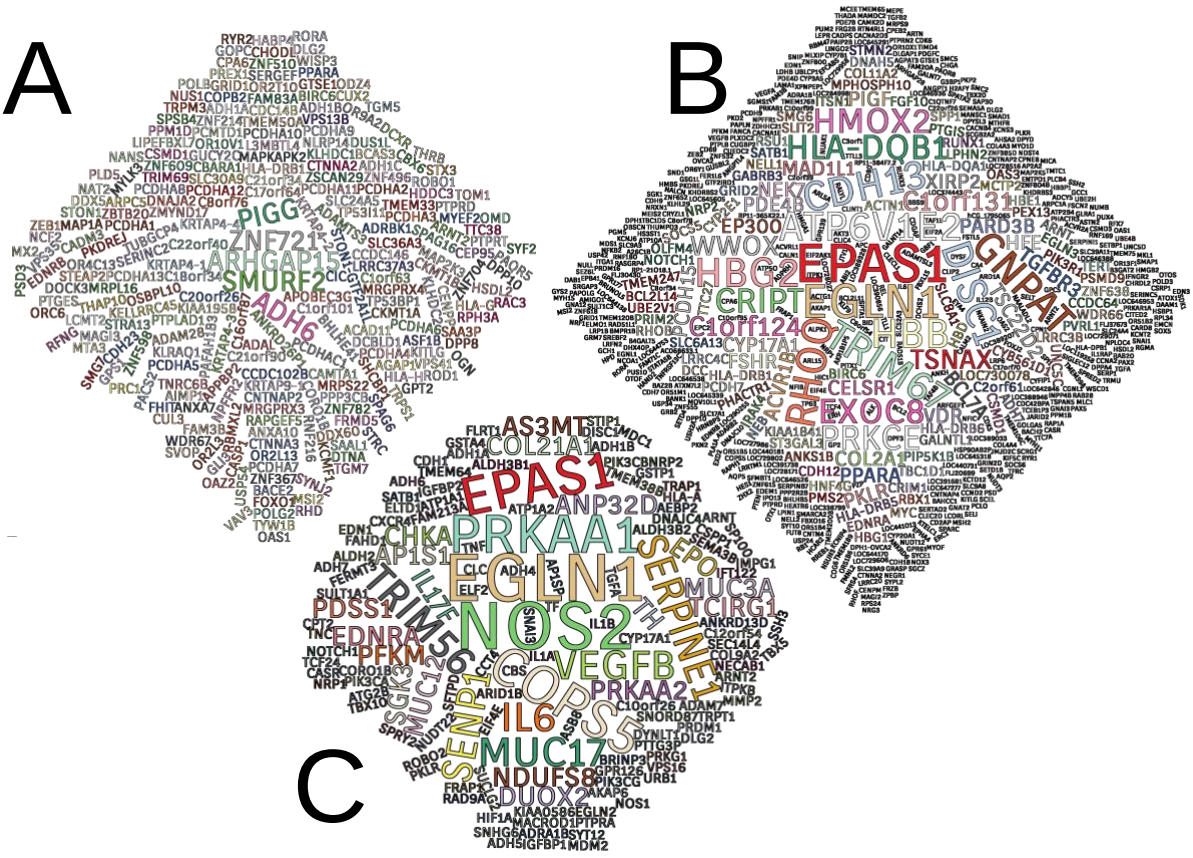


Figure 0.2: Figure from Yu et al. (2022) depicting word clouds of genes under positive selection in Ethiopian (A), Tibetan (B), and Andean (C) high-altitude populations. Text size is indicative of the number of times a top selection candidate gene is mentioned.

This evolutionary convergence also can be seen at the phenotypic level. For example, both Tibetans and Ethiopians exhibit [Hb] levels similar to those found at sea level (Beall, 2006; Beall et al., 2002). Although it's unclear whether reduced [Hb] is the target of natural selection in these cases, more recent work by Beall et al. (*In Revision*) suggests that having an intermediate [Hb] level (similar to those found at sea level) predicts greater reproductive success at high altitude. Similarly, Lawrence et al. (2024) found an association between a putatively adaptive variant of *EPAS1* and lower [Hb] in Andeans, suggesting that lower [Hb] is also adaptive in Andean highlanders. In contrast to these examples of convergence, Tibetans and Andeans differ in their ventilatory responses to hypoxia (Beall et al., 1997). The HVR, described in much more detail in Chapter 2, is the reflexive increase in ventilation in response to hypoxia (Powell, F. L. et al., 1998). Andeans generally have a lower HVR than Tibetans (Beall, 2007; Beall et al., 1997), and it has been speculated that this "blunted" ventilation may be a driver for increased [Hb] in Andeans (Beall, 2007; Heinrich et al., 2020). No studies of ventilation have been conducted in Ethiopians to date.

These high-altitude populations are not the only ones that reside or visit high elevations. Every year, millions of individuals travel to high altitude to either move, work, or recreate (Tremblay and Ainslie, 2021). These individuals face the same physiological pressures at high altitude as the highland populations, but are generally less well-equipped for them lacking hundreds of generations of evolution in their ancestry. However, just like highland populations, there is a considerable amount of variation in how these individuals respond to high altitude (Oeung et al., 2023; Yu, J. J. et al., 2022). In general, when sea-level residents travel to high altitude, they exhibit an increase in ventilation, increased [Hb], increased cerebral blood flow, and

cardiac changes (summarized in Fig. 0.3). However, these are all dependent on how much time they spend at high altitude, as each physiological change has its own time scale (Fig. 0.3).

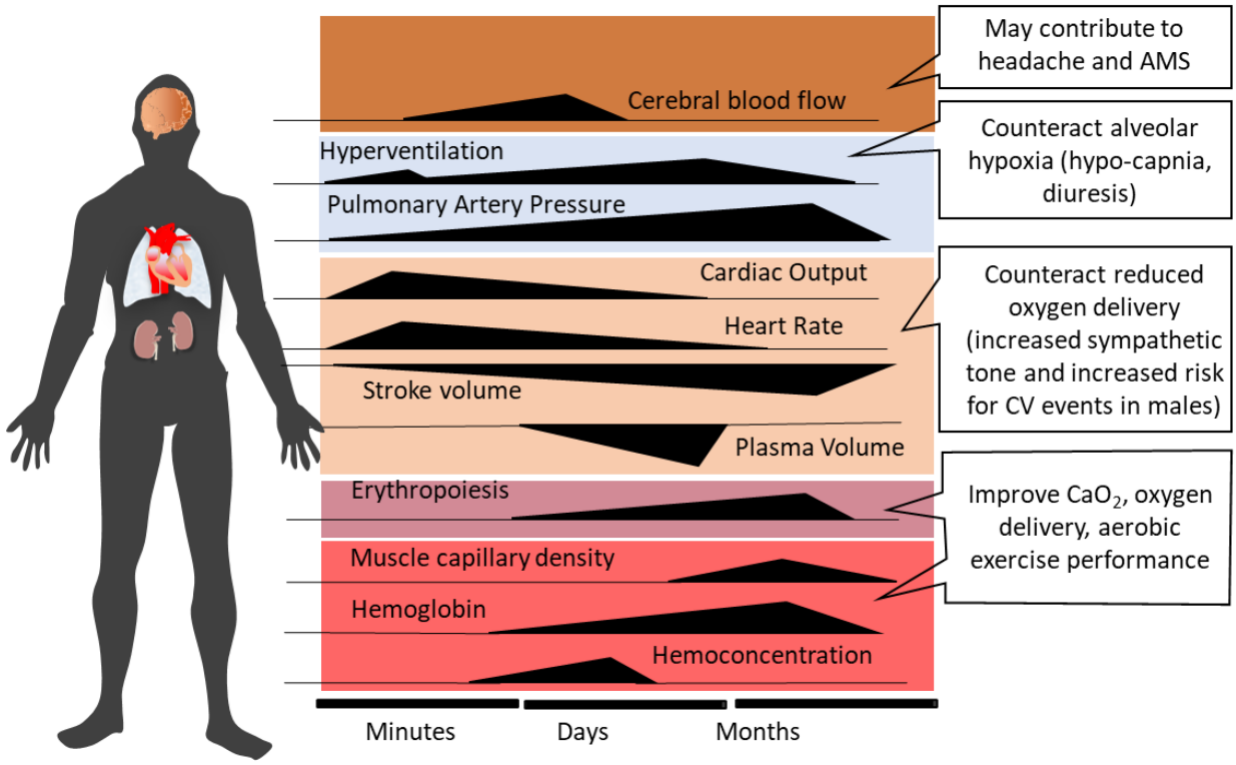


Figure 0.3: Figure from Mallet et al. (2023) summarizing physiological changes that occur during high-altitude acclimatization over varying time domains.

Understanding these varying responses to hypoxia across and within different populations is key to understanding human health. Oxygen is critical for human life and many diseases are governed by oxygen homeostasis, both at sea level and at high altitude (Fig. 0.4). The variability seen in the human response to hypoxia across different populations affords opportunities to investigate the relationship between the human body and oxygen from a molecular to a whole-organism level.

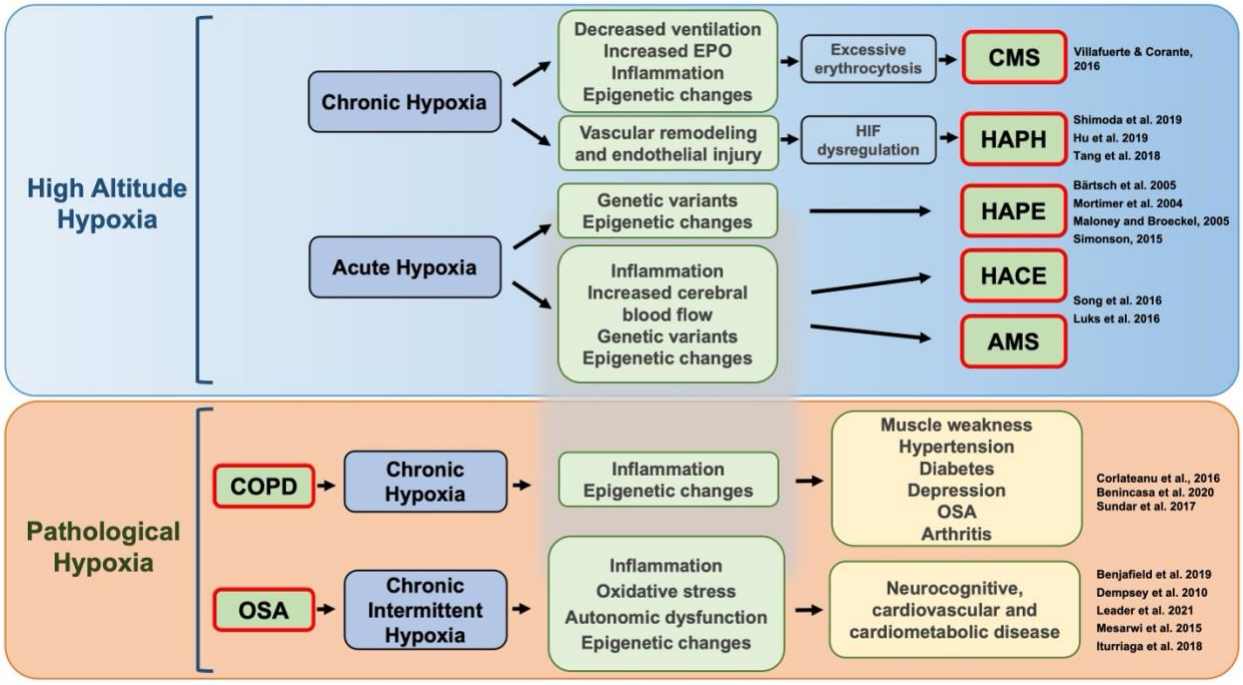


Figure 0.4: Figure from Yu et al. (2022) summarizing human diseases that are governed by oxygen homeostasis at both sea level and high altitude.

This dissertation explores the human response to high-altitude hypoxia through various lenses and distinct populations. The first chapter focuses on high-altitude adaptation in Andeans, utilizing a multi-omics analysis that spans together genetic, proteomic, and metabolomic data to predict physiological phenotypes related to hypoxia response. The second chapter focuses on high-altitude adaptation in Tibetans by characterizing the HVR in pre- and post-menopausal Tibetan women, along with exploratory genetic analyses identifying regions of the genome associated with ventilation and genes under positive selection that play a role in control of breathing. The third and last chapter focuses on sea-level residents acclimatizing to high altitude, summarizing a preliminary, randomized, double-blinded, placebo-controlled clinical trial that tests the ability of a probiotic to improve acclimatization to high altitude in a Latino population. Overall, the

dissertation provides insights into the human response to hypoxia in different contexts and in different populations.

Chapter 1 A multi-omics analysis of high-altitude adaptation in Andean highlanders

Abstract

High-altitude environments present significant physiological challenges due to hypobaric hypoxia, which leads to lower oxygen availability. Human populations residing at high altitudes for hundreds of generations, such as Andean highlanders, have developed unique adaptations in response to these environments. This study investigated the molecular mechanisms behind these adaptations by conducting a multi-omics analysis combining genomics, proteomics, and metabolomics. We collected phenotype data, including physiological, hematological, metabolic, and ventilatory measurements, from up to 296 Quechua Andean highlanders from Cerro de Pasco, Peru (4340 m). Within subsets of these individuals, we identified and genotyped 13 single-nucleotide polymorphisms SNPs under positive selection and analyzed plasma samples using a cardiometabolic proteomics panel and untargeted metabolomics. A Random Forest model identified influential metabolites for phenotype prediction, and linear mixed models determined significant multi-omic predictors while controlling for age, sex, body-mass index, and smoking status. We identified two genotypes, 37 proteins, and 338 metabolites that significantly predict at least one phenotype. Our findings provide insight into the genetic, proteomic, and metabolomic basis of high-altitude adaptation. This comprehensive multi-omics approach offers a valuable framework for future studies on the mechanisms underlying human adaptation to high altitude.

Introduction

High altitude environments present unique challenges to human physiology due to their extreme conditions. Chief among these challenges is hypobaric hypoxia, where there is less oxygen available at high elevations due to reduced atmospheric pressure. In response to this physiological

stressor of hypoxia, human populations that have resided at high altitude (>2,500 m) for hundreds of generations have developed various adaptation strategies to cope with high-altitude hypoxia (Simonson, 2015; Yu, J. J. et al., 2022). These adaptations begin at the molecular level with changes in the genome and culminate in distinct physiological traits that are beneficial at the whole-organism level, yet exactly how these genetic changes result in advantageous phenotypes is often unclear.

In the Andes, highlanders have occupied high altitude regions since at least 11,000 years ago (Nakatsuka et al., 2020; Rademaker et al., 2014). These Andean highlanders exhibit a range of physiological traits that are a result of their long-term occupation of high elevations and natural selection, including increased hemoglobin concentration (Beall, 2007), increased lung capacity (Brutsaert, T. D. et al., 2000; Brutsaert, T. D. et al., 1999), greater birth weight (Julian et al., 2011; Julian et al., 2009), and blunted ventilatory responses to hypoxia (Beall, 2007; Heinrich et al., 2020). These traits generally characterize the population, but importantly so, as there is also considerable variation of these traits within the population, affording the opportunity to investigate how genetic changes contribute to phenotypes related to high-altitude adaptation.

The advent of next-generation sequencing technologies have allowed genetic studies of larger scales in highland populations (Beall et al., 2010; Bigham et al., 2009; Simonson et al., 2010; Yi, X. et al., 2010). Many of these studies focused on identifying regions of the genome under positive selection in various high-altitude populations. In Andeans, Bigham et al. (2010) found evidence of natural selection occurring within the hypoxia inducible factor (HIF) pathway. A later study identified single-nucleotide polymorphisms (SNPs) within the genes *protein kinase, AMP-activated, alpha 1* (*PRKAA1*, encodes for α1 catalytic subunit of adenosine monophosphate kinase AMPK) and *endothelin receptor type A* (*EDNRA*) associated with birth weight and under

positive selection (Bigham et al., 2014). Crawford et al. (2017) utilized whole-genome sequencing to identify genes under the strongest selection, which were characterized as genes involved in cardiovascular development and not in the HIF pathway. More recent studies have explored specific mutations, with several studies identifying a missense mutation within the *endothelial PAS domain protein 1* (*EPAS1,* encodes for HIF2-α subunit) gene under positive selection and exhibiting functional effects and associations with hematocrit and exhaled nitric oxide (NO) in Andean highlanders (Eichstaedt et al., 2017; Jorgensen et al., 2023; Lawrence et al., 2024).

While these genetic studies have provided valuable insights into Andean high-altitude adaptation, they do not capture the full complexity of how these adaptations manifest at the physiological level. This gap can potentially be filled by other "-omics" approaches, such as transcriptomics, proteomics, and metabolomics. While these other omics have been utilized to study high-altitude adaptation and acclimatization in other populations, there is a lack of omics studies in Andean highlanders, as depicted by Figure 1, adjusted from Yu et al. (2022). Additionally, none of these studies combine various omics together into a multi-omics analysis, which can offer a more comprehensive understanding of the processes underlying biological phenomena. Often used to characterize disease states such as cancer (Krug et al., 2020; Parra, E. R. et al., 2024), multi-omics analyses offer a comprehensive method to link molecular changes to phenotypic outcomes. By understanding how changes at the genetic level translate into observable phenotype differences, we can gain insights into the mechanisms of health, disease, and adaptation. Despite the potential of multi-omics approaches, they have never been applied in an integrated manner using the same samples to explore phenotypes related to high-altitude adaptation.

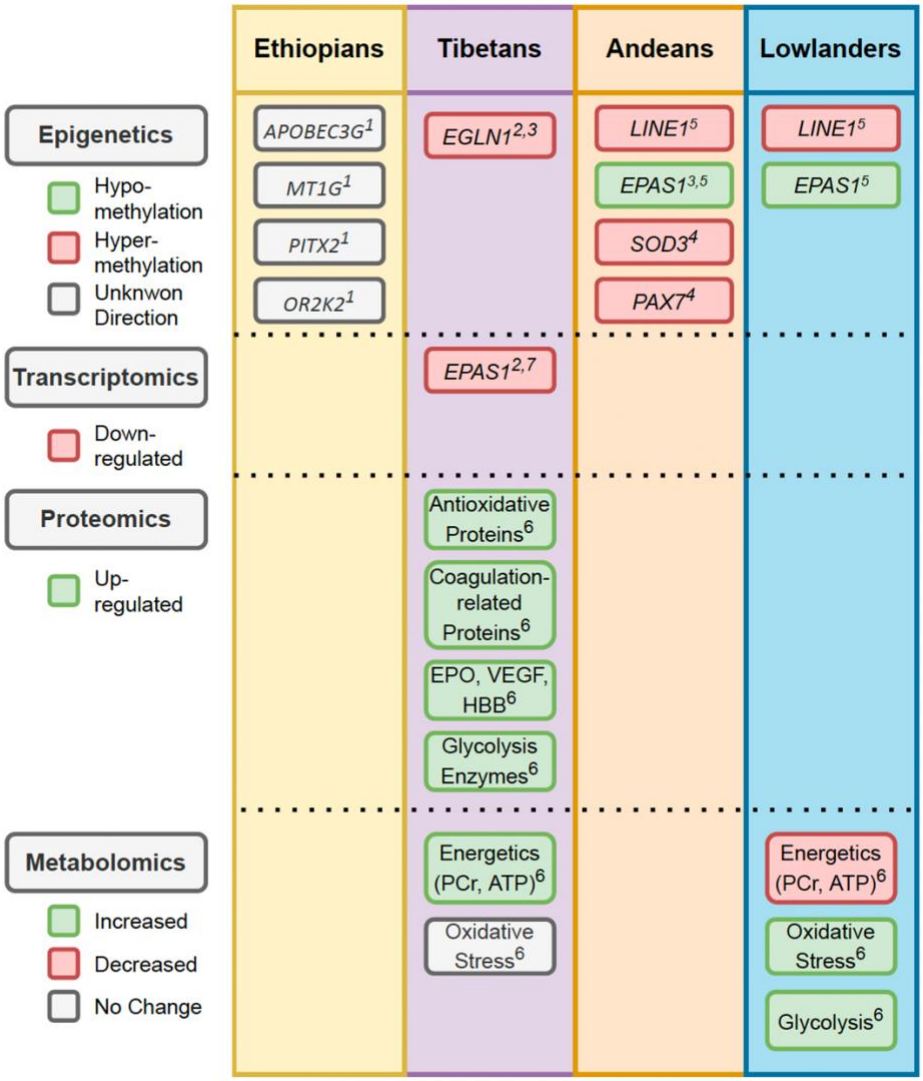


Figure 1.1: Figure adapted from Yu et al. (2022) summarizing -omics studies conducted in high-altitude populations and lowlanders who visit high altitude (>3600 m). Superscript references are 1) (2012) ; 2) Peng et al. (2017); 3) Julian et al. (2017); 4) Childebayeva et al. (2021); 5) Childebayeva et al. (2019); 6) Horscroft et al. (2017); 7) Xin et al. (2020).

In this study, we combine genomics, proteomics, and metabolomics from plasma samples in Andean highlanders to conduct a multi-omics analysis to determine predictors of phenotypes related to high-altitude adaptation. We utilize the results of a positive selection scan from 40 whole-genome sequences (Gu et al., *in preparation*) to identify 13 SNPs for further genotyping in

242 Andean highlanders, a cardiometabolic proteomics panel measuring 92 proteins, and untargeted metabolomics to predict 17 phenotype measurements related to basic physiology, hematology, metabolism, and ventilation. We hypothesized that various elements of these different omics would significantly predict proportions of phenotypes related to high-altitude adaptation.

Methods

Study participants

This study was approved by the University of California, San Diego Human Research Protection Program (140235, 171772) and the Universidad Peruana Cayetano Heredia Institutional Ethics Committee for Humans (59285). Participants 18-70 years of age were recruited by word of mouth and flyers, and measurements were collected during five trips to Cerro de Pasco, Peru (4340 m), from August 2015 to March 2019. Both males and non-pregnant females were included. Participants were deemed eligible if they self-reported their ancestry as Quechua Andean, both maternal and paternal grandparents had resided at high altitude, they hadn't traveled to lower elevation (<3000 m) for more than seven days in the previous six months, and if they self-reported no history of pulmonary, cardiovascular, or renal disease (besides Chronic Mountain Sickness). After determining eligibility for the study, participants received and signed a consent form in Spanish. Demographic information for participants is provided in Table 1.

Table 1.1: Demographic and physiological measurements of study participants.

Characteristic	N	Mean ± SD
Age (years)	387	41 ± 13
Sex	–	–
Female	78 (27%)	–
Male	211 (73%)	–
BMI (kg/m^2)	201	26.1 ± 3.6
Smoker	–	–
No	244 (86%)	–
Yes	41 (14%)	–
Hematocrit (%)	189	56 ± 8
SpO_2 (%)	296	86.3 ± 4.0
Resting HR (bpm)	215	71 ± 11
Systolic blood pressure (mmHg)	200	112 ± 14
Diastolic blood pressure (mmHg)	200	76 ±10
Glucose (mg/dL)	133	103 ± 45
Insulin (mIU/mL)	133	10.6 ±7.8
Cholesterol (mg/dL)	133	173 ± 40
HDL (mg/dL)	133	43 ± 12
LDL (mg/dL)	133	101 ± 32
Triglycerides	133	147 ± 86
Ferritin (mg/dL)	126	146 ± 196
Iron (µg/dL)	126	115 ± 46
Transferrin (mg/dL)	126	286 ± 45
HVR ($\Delta\dot{V}_I / \Delta SpO_2$)	105	0.10 ± 0.19
HCVR ($\Delta\dot{V}_I / \Delta ETCO_2$)	105	3.33 ± 16.74
Hypercapnic HVR ($\Delta\dot{V}_I / (\Delta ETCO_2 \times \Delta SpO_2)$)	105	0.32 ± 0.54

BMI, body mass index; SpO_2, oxygen saturation measured by pulse oximetry; HR, heart rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HVR, hypoxic ventilatory response; $\Delta\dot{V}_I$, minute ventilation; HCVR, hypercapnic ventilatory response; $ETCO_2$, end-tidal carbon dioxide; SD, standard deviation.

Whole-genome sequencing and positive selection analyses

Forty high-coverage whole-genome sequences from unrelated Quechua Andean men and women with self-identified high-altitude ancestry were analyzed using the Composite of Multiple Signals test (Grossman, S. R. et al., 2010) to determine regions of the genome under positive selection (summarized in Gu et al, *in preparation*). Regions identified as under positive selection were further analyzed using the integrated selection of allele favored by evolution (iSAFE) test (Akbari et al., 2018) to determine genetic variants within these regions that have high potential for driving the positive selection signal at that locus (summarized in Gu et al, *in preparation*). 13 genetic variants were selected from these iSAFE results for further genotyping in a larger cohort of Andean highlanders based off functional predictive scores such as the CADD score (Rentzsch et al., 2019), existing literature, and differences in allele frequencies compared to other populations (summarized in Table 2).

Table 1.2: Summary of 13 genotypes identified for further analysis in 242 Andean highlanders.

Gene	rsID	Region	CADD score	Allele frequency Andeans (%)*	Allele frequency world (%)*	Allele frequency admixed Americans (%)*
NOTCH1	rs61751489	exonic	7.42	29.13	1.88	13.79
PPARG	rs4684847	intronic	7.74	31.82	8.92	8.39
ICAM1	rs1799969	exonic	22.9	43.18	10.56	19.24
PRKAA1	rs59133000	intergenic	13.0	12.81	30.86	27.39
PRKAA1	rs3805490	intronic	1.14	80.17	21.46	37.70
F5	rs6018	exonic	13.0	23.35	6.11	9.77
EPAS1	rs4953354	intronic	0.877	45.66	22.03	28.65
PPARGC1A	rs3736265	exonic	18.2	82.64	6.56	9.43
PPARG	rs1801282	exonic	16.5	31.82	11.30	10.92
NOS3	rs7830	intronic	6.66	79.67	34.76	54.75
EGLN1	rs1769793	intergenic	0.924	60.58	32.93	44.85
ADRA1A	rs1048101	exonic	19.8	43.2	48.3	51.1
ADRA1A	rs2229125	exonic	27.2	86.8	2.46	5.61

*Reported by gnomAD (Chen, S. et al., 2024)

Genotyping

Venous blood samples were collected from 242 participants into EDTA tubes (BD Vacutainer, BD, Franklin Lakes, NJ, USA) and centrifuged to separate plasma, buffy coat, and red blood cells. The buffy coat was extracted and stored in a cell lysis solution at room temperature (Gentra Puregene Blood Kit, Qiagen, Germantown, MD, USA), and remaining samples were stored at -80C. Samples were shipped to University of California, San Diego where genomic DNA was isolated from the cell lysis solution and purified (Gentra Puregene Blood Kit, Qiagen, Germantown, MD, USA). DNA working stock plates of diluted concentrations (5 ng/ul) were created and DNA quality was verified (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA USA, 260/280 absorbance ratios of 1.8 to 2.0 and 260/230 absorbance ratios of 2.0 to 2.2). Targeted SNV genotyping was completed with Applied Biosystems TaqMan SNV Genotyping

Assays (Thermo Fisher Scientific, Waltham, MA, USA) and quantitative real-time polymerase chain reactions were conducted with a QuantStudio 3 (Applied Biosystems, Foster City, CA, USA). Genotyping calls were completed with the Applied Biosystems Genotyping Analysis Module (Applied Biosystems, Foster City, CA, USA).

Phenotype measurements

Phenotype measurements were collected during five trips to Cerro de Pasco, Peru (2015-2019). Hematocrit was measured from either venous blood using capillary tubes or a fingertip puncture using microcentrifugation. SpO_2 and resting heart rate were measured by pulse oximetry (Nellcor N-560, Medtronic, MN, USA). Blood pressure was measured with an electronic cuff (UA-767 Plus; A&D Medical, Japan) and three separate measurements were taken with the participant at rest for at least five minutes and averaged. Glucose, insulin, cholesterol, triglycerides, and iron measurements were taken from fasted blood serum samples. Ventilatory measurements were taken as previously described (Heinrich et al., 2020). A summary of phenotype measurements is provided in Table 1.

Proteomics

Plasma samples from a subset of 88 participants were analyzed to quantify relative abundance of 92 proteins known to be biomarkers of cardiometabolic function (Olink Target 96 Cardiometabolic Panel, Olink, Uppsala, Sweden) listed in Supplementary Table 1. The Olink panel utilizes a Proximity Extension Assay to bind pairs of oligonucleotide-labelled antibodies to their target protein, which are then extended by a DNA polymerase and quantified by real-time PCR

(Assarsson et al., 2014). The data are reported as Normalized Protein expression (NPX) values which are on the log2 scale and relative to the median of the intensity value for that plate.

Metabolomics

Plasma samples from a subset of 111 participants were analyzed to quantify the abundance of previously identified and unidentified metabolites using documented methodology (Lagerborg et al., 2019; Watrous et al., 2019). In brief, directed, non-targeted liquid chromatography-mass spectrometry (LC-MS) methods were utilized to identify small, polar, bioactive metabolites likely to be involved in cellular signaling, including free fatty acids, eicosanoids, oxylipins, bile acids, and fatty acid esters of hydroxy fatty acids. Mass spectrometry data were then extracted using image processing and machine learning based spectral optimization and then processed using in-house R scripts. Results were then annotated using in-house internal standards for MZ/RT and MS/MS matching.

Statistical analysis

Metabolomic, proteomic, and genotypic variability was compared against 18 phenotypes (hematocrit, hemoglobin concentration, SpO_2, systolic blood pressure, diastolic blood pressure, glucose concentration, insulin concentration, cholesterol, HDL, LDL, triglyceride concentration, ferritin concentration, iron concentration, transferrin concentration, hypoxic ventilatory response, hypercapnic ventilatory response, and hypoxic hypercapnic ventilatory response). A Random Forest model with 100,000 trees identified metabolites which most influenced the ability of the random forest model to predict each phenotype (phenotype ~.; Fig. 2). An influential metabolite was defined as having a mean decrease accuracy greater than the average, plus one standard

deviation of mean decrease accuracy. A linear mixed model identified proteomic signatures, genotypes, and influential metabolites that significantly predicted each phenotype while controlling for age, sex, BMI, and smoker status as random effects (phenotype ~ multi-omic signature + (1|sex) + (1|smoker) + (1|age) + (1|BMI); Fig. 2). To control for age and BMI, each was aggregated at increments of decades and five, respectively. Genotype allele counts were treated as a factor, and metabolites were $\log_{10}$ transformed to approximate gaussian distribution prior to the informatic pipeline. All p-values were false-discovery rate corrected using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). All multi-omic analyses were run in R (v.3.6.1) using the following libraries: tidyverse, data.table, DescTools, readxl, randomForest, lme4, and MuMIn. The R code used for data analysis is available from Github (github.com/zquinlan/yuMultiomicAnalysis2024).

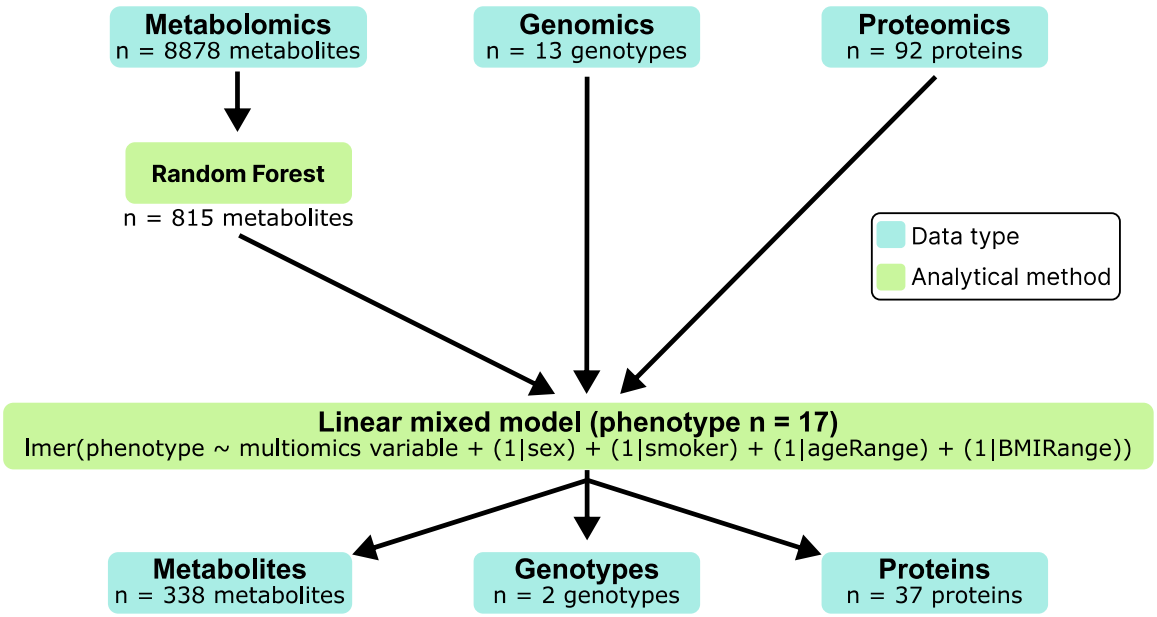


Figure 1.2: Graphical depiction of processing pipeline of multi-omics analysis. “lmer” is the R function for linear mixed-effects models.

Results

Phenotype measurements

Seventeen phenotypes were measured in up to 296 individuals from Cerro de Pasco, Peru. A summary of these phenotypes is provided in Table 1. These measurements include basic physiological measurements, hematological measurements, metabolism measurements, and ventilatory measurements (Table 1).

Positively selected genetic variants

Thirteen SNPs were genotyped in 242 individuals from Cerro de Pasco, Peru. The 13 SNPs are listed in Table 2 with information regarding location, allele frequencies, and CADD score. Most of the SNPs are more enriched within the Andean cohort compared to admixed Americans and the rest of the world, and many of the missense mutations have high CADD scores (Table 2).

Proteomics

Ninety-two proteins related to cardiometabolic function were quantified for relative abundance in 88 individuals from Cerro de Pasco, Peru. Names, Uniprot IDs of the proteins, and the genes encoding the proteins are provided in Supplementary Table 1.

Metabolomics

From the metabolomics analysis, 8,878 unique metabolites were identified and quantified in 111 participants from Cerro de Pasco, Peru. These results included previously identified and unidentified small, polar, bioactive metabolites.

Multi-omics analysis

A Random Forest model identified a total of 815 metabolites as influential for predicting at least one phenotype (further described in methods; Fig. 2). A linear mixed model identified two genotypes, 37 proteins, and 338 metabolites that were significant in relation to at least one of the phenotypes (LMER FDR p-value ≤ 0.0498; Fig. 2). A graphical summary of the results is provided in the form of a Circos plot (Krzywinski et al., 2009) in Figure 3 and significant genotype and proteomic predictors are listed in Table 3 with associated percentages of phenotype variance explained. A summary of 338 significant metabolite predictors that explained between 0.13% and 53.68% of the variation in at least one phenotype is provided in Table 4 (FDR corrected p-value ≤ 0.0498). All significant predictors and associated summary statistics are available in Supplemental File 1.1.

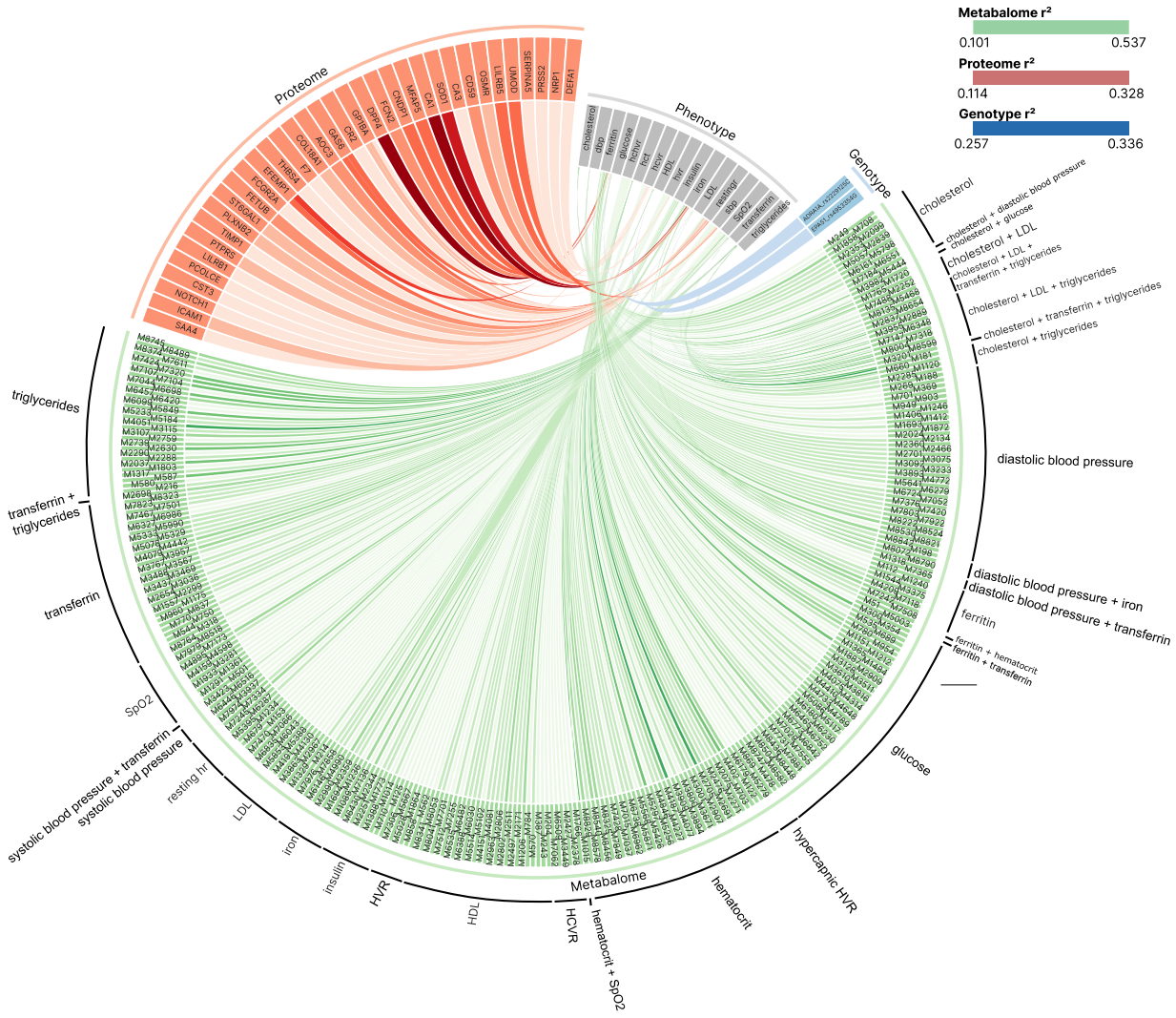


Figure 1.3: Circos plot of multi-omic data that explained at least 10% of the variance in a phenotype. Each omic is colored green, red, or blue to designate specific metabolomic features, proteins, or genotypes, respectively. Each omic is connected to the phenotype that is significantly explained with the link shaded based on the amount of variance that it explains (r^2). Metabolites are clustered based on which phenotypic response they explained.

Table 1.3: All significant genotype and proteomic predictors of at least one phenotype (FDR corrected p-value ≤ 0.0495).

Omics data type	**Phenotype**	**Omic feature**	**Percentage of variance in phenotype explained**	**FDR corrected p-value**
Genotype	Glucose	*ADRA1A*_rs2229125	15.79%	0.0258
Genotype	HDL	*EPAS1*_rs4953354	17.32%	0.0495
Proteomics	Hematocrit	SAA4	11.66%	0.00346
Proteomics	Resting HR	ICAM1	13.10%	0.0203
Proteomics	Resting HR	NOTCH1	11.75%	0.0258
Proteomics	Resting HR	CST3	10.80%	0.0344
Proteomics	Resting HR	PCOLCE	10.01%	0.0414
Proteomics	Resting HR	LILRB1	12.71%	0.0203
Proteomics	Resting HR	PTPRS	13.40%	0.0191
Proteomics	Resting HR	TIMP1	10.80%	0.0344
Proteomics	Resting HR	PLXNB2	14.75%	0.0134
Proteomics	Resting HR	ST6GAL1	13.37%	0.0167
Proteomics	Resting HR	DPP4	9.99%	0.0463
Proteomics	Resting HR	FETUB	16.42%	0.00626
Proteomics	Resting HR	TCN2	9.09%	0.0478
Proteomics	Resting HR	FCGR2A	12.06%	0.0242
Proteomics	Resting HR	EFEMP1	20.24%	4.18E-04
Proteomics	Resting HR	THBS4	10.98%	0.0490
Proteomics	Resting HR	F7	12.63%	0.0260
Proteomics	Resting HR	COL18A1	13.92%	0.0140
Proteomics	Resting HR	AOC3	16.65%	0.00521
Proteomics	Resting HR	GAS6	20.00%	0.00226
Proteomics	Resting HR	LILRB5	9.93%	0.0389
Proteomics	Systolic Blood Pressure	CR2	11.69%	0.0366
Proteomics	Systolic Blood Pressure	GP1BA	14.66%	0.0108
Proteomics	Glucose	DPP4	32.85%	0.00121
Proteomics	Glucose	FCN2	17.82%	0.00170
Proteomics	Glucose	CNDP1	19.96%	0.0237
Proteomics	Glucose	IGLC2	4.96%	0.0350
Proteomics	HDL	MFAP5	20.01%	0.0368
Proteomics	Ferritin	EFEMP1	23.04%	0.0191
Proteomics	Ferritin	GAS6	20.24%	0.0354
Proteomics	Iron	CA1	28.30%	0.00395
Proteomics	Iron	SOD1	27.19%	0.00395

Table 1.3 continued: All significant genotype and proteomic predictors of at least one phenotype (FDR corrected p-value ≤ 0.0495).

Omics data type	Phenotype	Omic feature	Percentage of variance in phenotype explained	FDR corrected p-value
Proteomics	Iron	GP1BA	14.02%	0.00776
Proteomics	Iron	CA3	11.75%	0.00697
Proteomics	Iron	CD59	17.63%	0.0368
Proteomics	Iron	OSMR	13.11%	0.0415
Proteomics	Iron	LILRB5	21.08%	0.0245
Proteomics	Transferrin	UMOD	19.49%	0.0396
Proteomics	HVR	SERPINA5	10.91%	0.0344
Proteomics	HCVR	PRSS2	12.30%	0.0354
Proteomics	HCVR	NRP1	11.69%	0.0414
Proteomics	HCVR	GP1BA	12.57%	0.0272
Proteomics	HCVR	DEFA1	11.09%	0.0425

Table 1.4: Summary of the variance explained for each phenotype by significant metabolomic predictors (FDR corrected p-value ≤ 0.0498).

Phenotype	Minimum explained variance	Maximum explained variance	Lowest p-value	N predictors
cholesterol	0.13%	43.94%	0.0368	38
diastolic blood pressure	11.36%	35.34%	0.0437	40
ferritin	18.90%	33.05%	0.0345	10
glucose	1.27%	45.72%	0.0463	45
hypercapnic HVR	11.64%	13.63%	0.0455	4
hematocrit	11.73%	53.68%	0.0358	36
HCVR	10.75%	17.06%	0.0425	6
HDL	1.33%	32.84%	0.0467	30
HVR	9.19%	19.98%	0.0397	7
insulin	5.99%	33.36%	0.0495	14
iron	7.30%	34.63%	0.0277	13
LDL	3.43%	27.70%	0.0463	28
resting HR	12.33%	37.72%	0.0335	8
systolic blood pressure	11.00%	16.38%	0.0487	4
SpO_2	9.70%	31.70%	0.0490	14
transferrin	3.17%	35.94%	0.0495	44
triglycerides	8.60%	51.50%	0.0429	48

Discussion

This study is the first multi-omics analysis investigating human adaptation to high altitude. We combined genetic, proteomic, and metabolomic data from Andean highlanders to investigate their contributions to phenotypes relevant to high-altitude adaptation, such as basic physiological measurements, hematology, metabolism, and ventilation. Using a combination of a Random Forest model to identify influential metabolites and linear mixed models, we identified two genotypes, 37 proteins, and 338 metabolites that significantly predicted at least one phenotype (FDR corrected p-value < 0.05). These results provide a basis for further investigation of these omic signatures for their role in high-altitude adaptation and future studies can focus on specific omic signatures to understand their connection to particular phenotypes. Additionally, this analysis provides a framework for future multi-omics analyses and can be expanded to include more data and data types, thereby increasing its effectiveness.

Phenotype measurements were collected over five separate trips to Cerro de Pasco, Peru. These 17 measurements range from the simple, such as resting HR, to the complex, such as ventilatory responses to hypoxia and hypercapnia (Table 1). Some of these phenotype measurements, such as hematocrit, SpO_2, and ventilation, are directly related to oxygen homeostasis while others, such as metabolism measurements, may be indirectly involved or altered by hypoxia responses. Regardless, all of these measurements are difficult to obtain from high-altitude fieldwork settings and require intense collaboration and logistical planning; the accumulation of these measurements in large enough sample sizes are what enable the depth of analysis in this study, in addition to the variability seen in these phenotypes within this population (Table 1).

The genotypes we included in the analysis are SNPs within genes under positive selection in Andean highlanders and have a strong likelihood of being functional mutations based off bioinformatic analyses and existing literature (Gu et al., *in preparation*; Table 2). The SNP rs2229125 explained 15.79% of the variance seen in glucose levels (Table 3). Rs2229125 is a missense variant within *Adrenoceptor Alpha 1A* (*ADRA1A*) at 86.8% allele frequency within the Andean cohort versus 2.46% worldwide and 5.61% in admixed Americans, respectively (Table 2). Additionally, rs2229125 has a CADD score of 27.2, indicating the genetic variant is within the top 0.7% likelihood of approximately 8.6 billion single-nucleotide variants of having a functional effect (Rentzsch et al., 2019). Adrenergic receptors play a key role in vasoconstriction and metabolic regulation (Insel, 1996; Perez, 2020). Although there are no studies associating rs2229125 with glucose levels, a study identified an association between a different SNP near *ADRA1A* and metabolic syndrome in an Italian population (Grassi et al., 2011), and another study identified ADRA1A as playing an essential role in adipocyte thermogenesis (Rahbani et al., 2022). Additionally, rs2229125 is an expression quantitative trait loci (eQTL) for *ADRA1A* and is associated with lower levels of expression of *ADRA1A* in adipose tissues (Beall et al., 2010; Eichstaedt et al., 2017; Jorgensen et al., 2023; Lawrence et al., 2024; Simonson et al., 2010; Yi, X. et al., 2010). Although the potential link between this SNP and glucose levels in Andean highlanders is unclear, our results indicate further investigation is warranted.

Rs4953354 is a mutation within the intronic region of *EPAS1* and explained 17.32% of the variance seen in HDL (Table 3). This SNP is also at a higher allele frequency in our Andean cohort at 45.66% than the rest of the world at 22.03% and admixed Americans at 28.65% (Table 2). *EPAS1*, which encodes for HIF2-α, a master regulator of hypoxia response pathways, has been identified to be under positive selection in both Andean and Tibetan highlanders (Beall et al., 2010;

Eichstaedt et al., 2017; Jorgensen et al., 2023; Lawrence et al., 2024; Simonson et al., 2010; Yi, X. et al., 2010). This particular SNP had been previously identified in positive selection scans in Tibetans and Sherpa and found to be associated with hemoglobin concentration in these groups (Hanaoka et al., 2012; Li, C. et al., 2019; Xu, J. et al., 2015). Additionally, rs4953354 is an eQTL for *EPAS1* and is associated with lower levels of *EPAS1* expression in blood (Võsa et al., 2021). However, how this SNP may potentially contribute to HDL levels is unclear.

The proteomics data we included in this analysis were markers of cardiometabolic function (Supplementary Table 1) and 37 unique proteins explained a significant amount of variance within at least one phenotype, including hematocrit, resting HR, systolic blood pressure, glucose levels, HDL, ferritin, iron, transferrin, HVR, and HCVR (Table 3). A cardiometabolic proteomics panel was chosen because chronic hypoxia can increase cardiometabolic risk and adaptation to high altitude may be protective against this risk (Miele et al., 2016). For example, Andean individuals with excessive erythrocytosis (EE) are at increased risk of cardiometabolic disease and may be considered less adapted to their hypoxic environment (Corante et al., 2018). Additionally, a positive selection scan by Crawford et al. (2017) indicated that genes related to cardiovascular health were under the strongest selective pressure. The proteins identified as significant predictors of at least one phenotype are listed in Table 3 and provide fertile starting grounds to investigate how these proteins may be impacting their respective phenotypes within the context of high-altitude adaptation.

The metabolomics analysis identified 8,878 unique metabolites, of which 338 were significant in relation to at least one phenotype (Fig. 2). All 17 phenotypes had significantly predictive metabolites, ranging from four to 48 metabolites (Table 4) and many of these metabolites predicted more than one phenotype (Fig. 3). While some of these metabolites have

been previously identified as particular free fatty acids and eicosanoids (Supplemental File 1.1), most of the metabolites are previously unidentified due to the LC-MS methods employed, which are specialized for identifying novel small, polar, bioactive metabolites likely to be involved in cellular signaling (Lagerborg et al., 2019; Watrous et al., 2019). Of the known metabolites, the free fatty acid dihomo-gamma-linolenic acid (DGLA) explained 51.5% of the variance in triglyceride levels and 43.9% in cholesterol (Supplemental File 1.1). DGLA potentially plays a role in inflammatory states (Mustonen and Nieminen, 2023) and supplementing with DGLA reduces thrombosis in humans (Kernoff et al., 1977). These potential roles of DGLA are relevant for Andean highlanders as they display some of the highest levels of EE in the world (Villafuerte et al., 2022) and EE is associated with increased thrombosis and inflammatory states (Corante et al., 2018).

Overall, this multi-omics analysis identified genetic, proteomic, and metabolomic predictors of phenotypes related to high-altitude adaptation. There were more metabolomic predictors than proteomic, and more proteomic predictors than genetic (Fig. 3). This may partially be due to relative abundance of predictors for each -omic, but may also be driven by how close each -omic lies to physiological phenotypes–metabolites, especially signaling molecules, lie closer to the phenotype in terms of biological relevance than proteins, and proteins closer than the genome (Sun, Y. V. and Hu, Y.-J., 2016). The effectiveness of multi-omics analyses strongly depend on the input data. In this case, the analysis would strongly benefit from larger datasets, but also inclusion of other data types, such as transcriptomics. Future studies can utilize the results of this analysis for further work elucidating the mechanisms behind some of these associations. For example, the SNP rs2229125 in *ADRA1A* can be investigated through functional genetic studies with tools such as precise genome editing, a method utilized in one of our previous studies

(Lawrence et al., 2024). The proteins shown to predict a portion of relevant phenotypes can be further explored to see how their varying levels contribute to healthy or diseased states. The identified metabolites, such as DGLA, can be tested for therapeutic effects and the previously unidentified metabolites can be structurally characterized to determine their chemical properties.

Conclusion

This is the first multi-omics analysis conducted in a high-altitude population. We combined genetic, proteomic, and metabolomic data from Andean highlanders to predict 17 phenotypes relevant to high-altitude adaptation. We identified two genotypes, 37 proteins, and 338 metabolites that significantly explained the observed variance at least one of the phenotypes. This analysis provides a framework for future multi-omic analyses and can be built upon to include other forms of -omics, such as transcriptomics, by utilizing a similar model with more omic inputs.

Acknowledgements

Chapter 1, in full, is currently being prepared for submission for publication of the material. Yu, James J.; Quinlan, Zachary A.; Gu, Wanjun; Li, Jane; Anza-Ramirez, Cecilia; Vizcardo-Galindo, Gustavo; Macarlupu, Jose-Luis ; Figueroa-Mujica, Rómulo; Bermudez, Daniela; Corante, Noemi; Cole, Amy; Gaio, Eduardo; Mercader, Kysha; Long, Tao; Alotaibi, Mona; Jain, Mohit; Heinrich, Erica C.; Villafuerte, Francisco C.; Simonson, Tatum S. The dissertation author was the primary investigator and author of this paper.

Supplemental Table 1.1: List of proteins assayed in proteomics cardiometabolic panel.

Uniprot ID	**Protein name**	**Gene name**
P42785	Lysosomal Pro-X carboxypeptidase	*PRCP*
P00915	Carbonic anhydrase 1	*CA1*
P05362	Intercellular adhesion molecule 1	*ICAM1*
O00533	Neural cell adhesion molecule L1-like protein	*CHL1*
Q15582	Transforming growth factor-beta-induced protein ig-h3	*TGFBI*
P17813	Endoglin	*ENG*
P55058	Phospholipid transfer protein	*PLTP*
P05543	Thyroxine-binding globulin	*SERPINA7*
P17936	Insulin-like growth factor-binding protein 3	*IGFBP3*
P20023	Complement receptor type 2	*CR2*
P05154	Plasma serine protease inhibitor	*SERPINA5*
O75015	Low affinity immunoglobulin gamma Fc region receptor III-B	*FCGR3B*
P24592	Insulin-like growth factor-binding protein 6	*IGFBP6*
P12830	Cadherin-1	*CDH1*
P13501	C-C motif chemokine 5	*CCL5*
Q16627	C-C motif chemokine 14	*CCL14*
P22749	Granulysin	*GNLY*
P46531	Neurogenic locus notch homolog protein 1	*NOTCH1*
P19021	Peptidyl-glycine alpha-amidating monooxygenase	*PAM*
P04070	Vitamin K-dependent protein C	*PROC*
P01034	Cystatin-C	*CST3*
P13591	Neural cell adhesion molecule 1	*NCAM1*
Q15113	Procollagen C-endopeptidase enhancer 1	*PCOLCE*
Q8NHL6	Leukocyte immunoglobulin-like receptor subfamily B member 1	*LILRB1*
P08581	Hepatocyte growth factor receptor	*MET*
Q14767	Latent-transforming growth factor beta-binding protein 2	*LTBP2*
P16871	Interleukin-7 receptor subunit alpha	*IL7R*
P19320	Vascular cell adhesion protein 1	*VCAM1*
P14151	L-selectin	*SELL*
P03951	Coagulation factor XI	*F11*
P49747	Cartilage oligomeric matrix protein	*COMP*
P22748	Carbonic anhydrase 4	*CA4*
Q13332	Receptor-type tyrosine-protein phosphatase S	*PTPRS*
P11226	Mannose-binding protein C	*MBL2*
P01033	Metalloproteinase inhibitor 1	*TIMP1*
Q9Y5C1	Angiopoietin-related protein 3	*ANGPTL3*

Supplemental Table 1.1 Continued: List of proteins assayed in proteomics cardiometabolic panel.

Uniprot ID	**Protein name**	**Gene name**
Q06141	Regenerating islet-derived protein 3-alpha	*REG3A*
P00441	Superoxide dismutase [Cu-Zn]	*SOD1*
P15529	Membrane cofactor protein	*CD46*
P11215	Integrin alpha-M	*ITGAM*
P24821	Tenascin	*TNC*
P14543	Nidogen-1	*NID1*
Q9BXR6	Complement factor H-related protein 5	*CFHR5*
Q14515	SPARC-like protein 1	*SPARCL1*
O15031	Plexin-B2	*PLXNB2*
Q9H1U4	Multiple epidermal growth factor-like domains protein 9	*MEGF9*
P03950	Angiogenin	*ANG*
P15907	Beta-galactoside alpha-2,6-sialyltransferase 1	*ST6GAL1*
P27487	Dipeptidyl peptidase 4	*DPP4*
P05451	Lithostathine-1-alpha	*REG1A*
Q16769	Glutaminyl-peptide cyclotransferase	*QPCT*
Q15485	Ficolin-2	*FCN2*
Q9UGM5	Fetuin-B	*FETUB*
P23141	Liver carboxylesterase 1	*CES1*
Q9NQ79	Cartilage acidic protein 1	*CRTAC1*
P20062	Transcobalamin-2	*TCN2*
P07478	Trypsin-2	*PRSS2*
P32942	Intercellular adhesion molecule 3	*ICAM3*
P35542	Serum amyloid A-4 protein	*SAA4*
Q96KN2	Beta-Ala-His dipeptidase	*CNDP1*
P12318	Low affinity immunoglobulin gamma Fc region receptor II-a	*FCGR2A*
O14786	Neuropilin-1	*NRP1*
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	*EFEMP1*
Q96H15	T-cell immunoglobulin and mucin domain-containing protein 4	*TIMD4*
Q12884	Prolyl endopeptidase FAP	*FAP*
P35590	Tyrosine-protein kinase receptor Tie-1	*TIE1*
P35443	Thrombospondin-4	*THBS4*
P08709	Coagulation factor VII	*F7*
P07359	Platelet glycoprotein Ib alpha chain	*GP1BA*
Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1	*LYVE1*
P07451	Carbonic anhydrase 3	*CA3*

Supplemental Table 1.1 Continued: List of proteins assayed in proteomics cardiometabolic panel.

Uniprot ID	**Protein name**	**Gene name**
Q03167	Transforming growth factor beta receptor type 3	*TGFBR3*
P59665	Neutrophil defensin 1	*DEFA1_DEFA1B*
P13987	CD59 glycoprotein	*CD59*
O95445	Apolipoprotein M	*APOM*
Q99650	Oncostatin-M-specific receptor subunit beta	*OSMR*
Q8N423	Leukocyte immunoglobulin-like receptor subfamily B member 2	*LILRB2*
P07911	Uromodulin	*UMOD*
P55774	C-C motif chemokine 18	*CCL18*
P39060	Collagen alpha-1	*COL4A1*
P80188	Neutrophil gelatinase-associated lipocalin	*LCN2*
P10721	Mast/stem cell growth factor receptor Kit	*KIT*
Q9BXJ1	Complement C1q tumor necrosis factor-related protein 1	*C1QTNF1*
Q16853	Membrane primary amine oxidase	*AOC3*
Q14393	Growth arrest-specific protein 6	*GAS6*
P0DOY2	Immunoglobulin lambda constant 2	*IGLC2*
Q13093	Platelet-activating factor acetylhydrolase	*PLA2G7*
P22105	Tenascin-X	*TNXB*
Q13361	Microfibrillar-associated protein 5	*MFAP5*
Q6EMK4	Vasorin	*VASN*
O75023	Leukocyte immunoglobulin-like receptor subfamily B member 5	*LILRB5*
P06681	Complement C2	*C2*

Chapter 2 Ventilatory and Heart Rate Responses to Hypoxia in Pre- and Post-menopausal ethnically Tibetan Women in Mustang, Nepal

Abstract

When humans are exposed to acute hypoxia, they increase their ventilation and heart rate to compensate for a decrease in available oxygen, respectively known as the hypoxic ventilatory response (HVR) and the hypoxic heart rate response (HHRR). Populations that have resided at high altitude for hundreds of generations exhibit distinct physiological responses to acute hypoxia due to evolution. Tibetans have been observed to have higher HVRs than other high-altitude residents, a potentially adaptive trait, but most of these studies have focused on males and none have studied the effects of menopausal status on HVR. Due to the known effect of reproductive hormones increasing HVR, we hypothesized that HVR and HHRR decrease with menopausal status in Tibetan women. To test this hypothesis, we measured the isocapnic HVR and HHRR in 366 Tibetan women residing in Mustang, Nepal (3500 m) and tested for associations with menopausal status. In order to identify genetic traits related to ventilation, we performed a genome-wide association study (GWAS) for ventilatory measurements, and a positive selection scan overlapped with a control of breathing candidate gene list. HVR and HHRR were higher in the pre-menopausal Tibetan women (0.34±0.24 and 0.911±0.383, respectively) than the post-menopausal Tibetan women (0.21±0.16 and 0.649±0.278, respectively; $p<0.0001$). The GWAS revealed 42 SNPs associated with $PetCO_2$ at the genome-wide significance level and one SNP with minute ventilation during hypoxia. The positive selection scan overlapped with a control of breathing candidate gene list identified *EPAS1*, *PPARG*, *CHCHD2*, and *EGLN1* as both under positive selection and playing a role in control of breathing. These data suggest Tibetan women experience a reduction in HVR after menopause. A non-blunted HVR is a hallmark of adaptation

in Tibetans, and physiological changes observed in Tibetan women after menopause may result in a loss of such traits.

Introduction

Humans ascending to high altitude face the physiological pressure of hypobaric hypoxia, breathing less available oxygen. The first physiological response to this decreased oxygen level (seconds to minutes) is an increase in ventilation termed hypoxic ventilatory response (HVR), produced by the activation of the peripheral chemoreceptors within the carotid body, that sense the decrease in arterial P_{O_2} (Iturriaga et al., 2021). Concurrently, an acute hypoxic event produces an increase in heart rate (Kronenberg and Drage, 1973; Vogel and Harris, C. W., 1967), or a hypoxic heart rate response (HHRR), triggered by a mechanism that includes activation of the adrenergic system (Lhuissier, F. J. et al., 2012; Richalet et al., 2023). The HVR and the HHRR produce a compensatory effect in response to hypoxia, aiming to maintain the necessary delivery and transport of oxygen to vital organs (Richalet et al., 2023; Teppema and Dahan, 2010). These ventilatory and cardiovascular responses are variable over time, among individuals, and among different populations, however the mechanisms underlying these differences are not completely understood (Pamenter and Powell, F. L., 2016; Powell, F. L. et al., 1998).

Many factors contribute to HVR variability. These include age (García-Río et al., 2007; Lhuissier, F. J. et al., 2012), differences in metabolism (Zwillich et al., 1977), body temperature (Gautier, 1996), circadian rhythms (Gerst et al., 2011), pathological factors (Dempsey and Smith, C. A., 2014), use of drugs (Teichtahl et al., 2005), pregnancy (Moore, L. G. et al., 1987), and hormonal status (Moore, L. G. et al., 1987). In addition, HVR variability may be attributed to genetic factors (Brutsaert, T. D. et al., 2005; Golder et al., 2005; Han, F. and Strohl, 2000;

Lancaster et al., 2020; Moya et al., 2024; Petousi et al., 2014; Song, D. et al., 2020), which is further supported by HVR differences observed between populations with distinct ancestries, ranging from histories at sea-level to high-altitude Andean and Tibetan populations (Beall et al., 1997; Brutsaert, T. D. et al., 2005; Goldberg et al., 2017; Oeung et al., 2023; Spicuzza et al., 2004). For example, individuals of Tibetan genetic ancestry have, on average, higher HVRs than individuals of Han Chinese or Andean genetic ancestry at high altitude (Beall, 2007; Beall et al., 1997; Zhuang, J. et al., 1993).

The majority of studies on ventilation have been performed in men travelling to high altitude (Forster et al., 1971; Hupperets et al., 2004; Sato et al., 1992; Weil et al., 1971; Weil et al., 1970; Zhuang, J. et al., 1993). Increasing the amount of HVR studies including women is critical because there may be contributors to ventilation variability that are either less pronounced or completely absent in men. For example, hormones may contribute to HVR variability (Moore, L. G. et al., 1987; Pickett et al., 1989; Regensteiner et al., 1989; Saaresranta and Polo, 2002); progesterone increases ventilation in both men and women and the combination of progesterone and estrogen may have a synergistic effect in increasing ventilation (Behan and Wenninger, 2008). In addition, HVR increases with pregnancy (Moore, L. G. et al., 1987). These observations have led to speculation on whether menstrual cycle affects HVR within females. Previous studies indicate the HVR varies with menstrual cycle within individuals, with higher HVRs observed during the luteal phase (Richalet et al., 2020; Schoene et al., 1981; White, D. P. et al., 1983), while other studies found no effect of menstrual cycle on rate of ventilatory acclimatization (Muza et al., 2001).

Furthermore, how menopausal status affects HVR in women is even less studied. Studying sea-level residents at resting conditions, Pokorski and Marczak (2003) did not find significant

differences in the HVR of elderly versus young women, and Lhuissier et al. (2012) found that HVR increases with age but that this increase stops in post-menopausal untrained women. In addition, other authors found that menstrual cycle did not impact the rate of ventilatory acclimatization (Muza et al., 2001), acute mountain sickness susceptibility is not affected by menstrual status (Gardner et al., 2024), and women living at high altitude have an earlier onset of menopause compared with women at sea level (Mateikaitė-Pipirienė et al., 2023). In studies of women with high-altitude ancestry, Andean pre-menopausal women are better protected against certain diseases caused by chronic hypoxia such as Chronic Mountain Sickness (CMS) (León-Velarde et al., 2000; León-Velarde et al., 1997) and this may partially be due to estrogen levels (Azad et al., 2021) and progesterone levels (León-Velarde et al., 2001), though how the HVR may contribute to the development of these diseases is unknown. No studies of the effects of menopause on ventilation or responses to hypoxia have been conducted in Tibetans.

The cardiovascular responses to hypoxia also show high degrees of variability between individuals. The responses are attributed to the activation of several mechanisms, including stimulation of central nervous system and arterial chemoreceptors, direct effects of hypoxia and hypocapnia on the heart and vasculature, modulation of the nervous system by secondary mechanisms such as pulmonary stretch afferents, alkalosis produced by hyperventilation, and increased secretion of catecholamine from the adrenal glands (Burgh Daly, de, 1997; Joyce and Wang, T., 2022). Specifically, exposure to acute hypoxia produces an increase of heart rate concomitant to an increased sympathetic activity (Hanada et al., 2003; Halliwill et al., 2003) and, in high-altitude hypoxia, the increase in heart rate is related to an increase in sympathetic activity and vagal withdrawal (Burgh Daly, de, 1997; Halliwill et al., 2003; Hansen, J. and Sander, 2003; Koller et al., 1988). At a population level, it is possible that genetic factors may contribute to

different HHRRs observed in humans with high-altitude ancestry history. For example, Zhou et al. (2008) studied Tibetans born and raised at high altitude (3700m) who migrated to sea level for four years and compared them with a lowland Han Chinese group. The authors exposed both groups to acute hypoxia in a hypobaric chamber (2 hours, simulating 3700m) and found that acute hypoxia induced a significant increase of heart rate in the Han Chinese but not in the Tibetan population (Zhou, Z.-N. et al., 2008). Additionally, Moya et al. (2024) observed lower HHRRs in Tibetans during poikilocapnic hypoxia at intermediate altitude (1300 m) compared to Han Chinese. However, these studies have been performed mainly in males, and the contribution of menopausal status on HHRR in Tibetan females has not been studied.

Thus, with the lack of information of high-altitude women's physiological responses to hypoxia and the contribution of menopausal status on these responses, we decided to study the HVR and HHRR in a older female Tibetan population residing at high altitude. We then conducted exploratory genetic analyses, including a genome-wide association study (GWAS) with HVR measurements and a positive selection scan overlapped with a control of breathing candidate gene list.

We hypothesize that Tibetan pre-menopausal women residing at high altitude show increased ventilatory and cardiac responses to acute hypoxia when compared to post-menopausal Tibetan women, and that ventilatory measurements from the overall cohort will associate with genotypes. To test this hypothesis, we measured HVR and HHRR with a modified re-breathing method maintaining isocapnia in 366 women of Tibetan ancestry in Mustang, Nepal, compared ventilatory and heart rate responses between pre-menopausal and post-menopausal Tibetan women, conducted a GWAS for ventilatory measurements, and a positive selection scan overlapped with a control of breathing candidate gene list.

Methods

Study participants

We recruited a total of 417 individuals who self-identified as ethnically Tibetan women between 46-86 years of age as previously reported (Ye et al., *In Preparataion*). All participants resided in Upper Mustang District, Nepal, in villages raging between 3500-4100 meters (11,550-13,530 feet) above sea level. Data collection was performed in temporary laboratories installed in three of the main area villages: Lo Manthang (3800 m, latitude, longitude 29º10'N, 83º57'E, Tsarang (3500 m; latitude, longitude 29º5'N, 83º56'E), and Ghami (3500 m; latitude, longitude 29º3'N, 89º53'E). We performed HVR in 376 of those participants, and from those 10 did not provided menopausal status information or did not fulfill physiological measurement requirements (listed below), which resulted in a total of 366 women included in this study. In one of the 366, we were not able to record heart rate information.

Anthropometric measurements

Anthropometric measurements, including height, weight, calculated body-mass index (BMI) and tibial length, were collected from all participants at the beginning of each study. Participants were also questioned about their menopausal status during these first measurements.

Physiological measurements

For physiological measurements of HVR and HHRR, participants sat in a chair, a nose clipped was placed to ensure mouth breathing, and they were asked to breathe through a mouthpiece (Hans Rudolph, Shawnee, KS) connected to our measurement system (Fig. 1A). The mouthpiece was connected to a one-way, rebreathing circuit with a breathing valve (2700 Series,

Large, Hans Rudolph) to produce a decrease in the fraction of inspired oxygen (F_{IO_2}) when the rebreathing circuit is closed to room air. The protocol consisted of one to three acute hypoxia events, and tidal volume (V_T), respiratory frequency (f_R), minute ventilation ($\dot{V}_I$), and heart rate (HR), oxygen saturation (SpO_2) and end-tidal CO_2 ($P_{ET}CO_2$) were collected (Fig. 1B) and used to calculate HVR and HHRR. In every trial, we recorded baseline measurements breathing room air for 2-3 minutes followed by a hypoxic stimulus (3-5 min) induced by rebreathing. A trial was considered successful if it produced a decrease of at least 10% in O_2 saturation from the values recorded in baseline conditions.

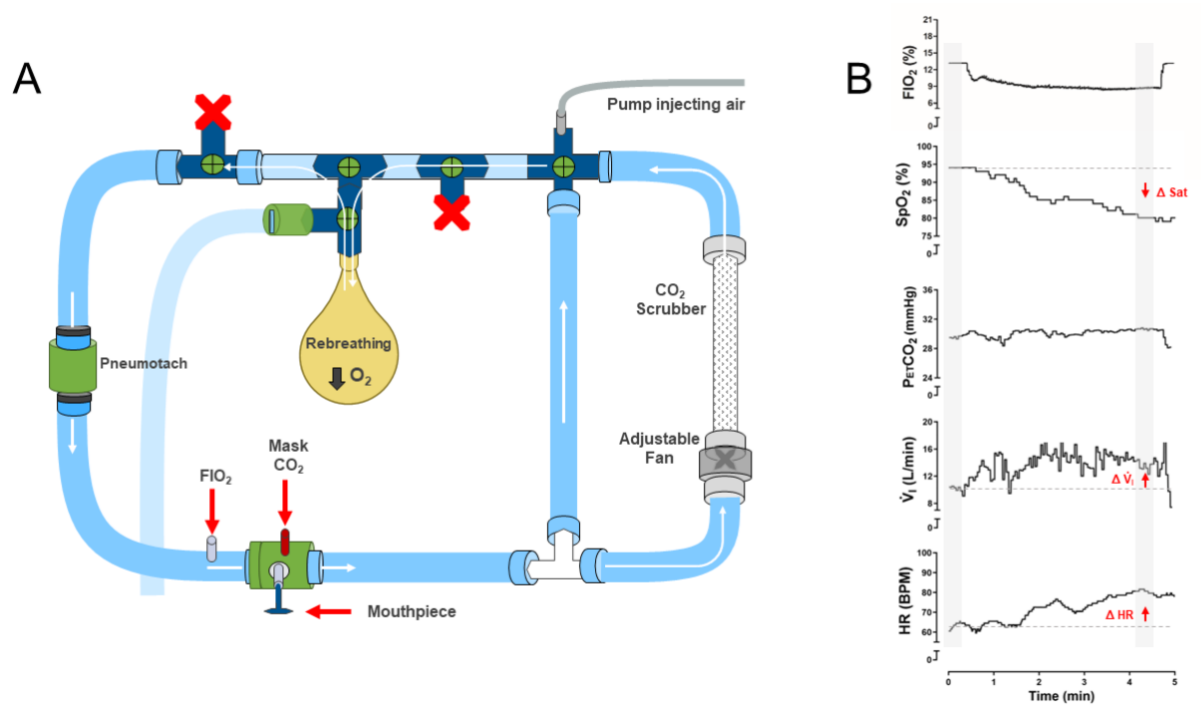


Figure 2.1: (A) Cartoon depiction of the HVR apparatus. We utilized a modified version of a re-breathing circuit due to inability to access gas tanks at the fieldwork sites. Red Xs depict where air flow is blocked, creating a re-breathing circuit. (B) Example recording of an isocapnic HVR measurement. The gray portions represent where average values were taken to quantify the HVR.

Initially, for baseline-room air recordings, a three-way valve upstream of the mask and a three-way valve downstream of the mask were opened to the atmosphere, allowing the volunteer to breathe room air. O_2 and CO_2 were continuously sampled from the breathing valve directly in front of the mouth by CO_2 (Model 17515A, VacuMed, Ventura, CA, USA) and O_2 (Model 17620, VacuMed) analyzers. Inspiratory flow was measured by a Fleisch pneumotachograph located upstream of the mask and connected to a carrier demodulator (model CD15, Validyne, Northridge, CA, USA). We recorded SpO_2 and HR during the hypoxic stimulus with a pulse oximeter using a surface probe placed on the forehead (Nellcor model N395, Medtronic, Minneapolis, MN, USA). All analog signals were processed through a PowerLab 8/30 (ADInstruments, Colorado Springs, CO, USA) and sent digitally to a laptop computer (HP Probook, HP Inc., Palo Alto, CA, USA). Raw data was recorded in LabChart 8 (ADInstruments) during a five-minute period (Fig. 1B).

To start the hypoxic stimulus, the 3-way valves were closed to produce a decrease in O_2 and a third 3-way valve connected to a weather balloon was simultaneously opened to increase the volume in the rebreathing circuit. The changes in O_2 and CO_2 were monitored continuously. Once the 10% desaturation was achieved, an air pump was used to add atmospheric air into the circuit. The hypoxic stimulus was maintained in isocapnic conditions with a CO_2 scrubber positioned in parallel to the rebreathing circuit. The amount of air going into the CO_2 scrubber was controlled with a regulating fan located just before the column with CO_2 scrubber. Hypoxic events that presented a change in $P_{ET}CO_2$ higher than 2 mmHg compared to its baseline measurement value were discarded. For calculation of HVR and HHRR, averages of the measured variables' values during a 30-seconds minimum period during room air baseline and hypoxic conditions were analyzed (gray areas in Fig. 1B). The HVR was calculated as the change in ventilation per decrease

in SpO2 ($\dot{V}_I$ / ΔSpO_2), and the HHRR was calculated as the change in heart rate per decrease in SpO_2 (HR / ΔSpO_2) during the period selected for HVR.

Genome-wide association study

We conducted a genome-wide association study (GWAS) using previously published data (available at https://datadryad.org/stash/dataset/doi:10.5061/dryad.bp46m) that contains approximately 3.5 M single nucleotide polymorphisms (SNPs) for this same cohort of women (Jeong et al., 2018). We tested these SNPs for genome-wide significance with physiological variables related to ventilation (HVR, HHRR, V_T in room air and hypoxia, $\dot{V}_I$ in room air and hypoxia, R_f in room air and hypoxia, SpO_2 in room air and hypoxia, and $P_{ET}CO_2$ in room air and hypoxia) using GEMMA v0.94.1 (Zhou, X. and Stephens, 2012). We included SNPs with a minor allele frequency ≥ 5% and performed a stepwise regression with covariates age, age^2, altitude, relative wealth, menopausal status, F_{IO_2} in room air, perfusion, and fingertip temperature, and selected the regression model with the lowest Akaike information criterion (AIC) values.

Positive selection scan

We employed the iSAFE algorithm (Akbari et al., 2018) to identify genomic regions exhibiting significant signals of natural selection. Genomic data from a larger cohort of 417 individuals that included all 366 individuals with HVR and HHRR measurements was segmented into five-megabase bins. Variants with an iSAFE score of 0.2 or higher were selected for further investigation. We generated a list of candidate genes with genomic regions exhibiting a signal of positive selection by annotating prioritized variant locations and including genes within 200 kilobases (kb) up- and down-stream. Finally, this list was cross-referenced with a control of

breathing candidate gene list (Supplementary Table 1) that was generated based on existing literature. The overlap of these lists is provided in Table 4.

Results

Anthropometric measurements

Anthropometric values for pre- and post-menopausal Tibetan women are listed in Table 1. We found significant differences between pre- and post-menopausal women for age, height, and weight. However, we did not find significant differences in BMI or tibial length between groups.

Table 2.1: Population characteristics of Tibetan women in Mustang, Nepal. Mean ± SD.

Parameter	**Total**	**Menstrual Status**		**t-test**
	(n)	Pre-menopause (n)	Post-menopause (n)	***P***
Age (years)	59 ± 9.0 (366)	49 ± 3.4 (66)	61 ± 8.4 (300)	****
Height (m)	1.50 ± 0.06 (366)	1.52 ± 0.05 (66)	1.49 ± 0.06 (300)	**
Weight (Kg)	48.0 ± 8.6 (366)	51.2 ± 7.4 (66)	47.5 ± 8.3 (300)	*
BMI (Kg/m^2)	21.27 ± 3.00 (366)	21.9 ± 2.8 (66)	21.1 ± 3.0 (300)	n.s.
Tibial Length (cm)	31.8 ± 1.91 (366)	32.1 ± 1.84 (66)	31.7 ± 1.92 (300)	n.s.

Students' t-test, **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$.

Hypoxic ventilatory responses

The reduction of P_{O_2} while maintaining constant $P_{ET}CO_2$ ensured isocapnic conditions and produced a significant decrease of SpO_2 and a significant increase of $\dot{V}_I$, V_T, R_f, and HR in all Tibetan women combined (Table 2). The pre-menopausal Tibetan women had higher $\dot{V}_I$ (12.6 ± 1.9 L/min) and SpO_2 (93.7 ± 2.5%) while breathing room air compared to the post-menopausal Tibetan women (11.5 ± 1.7 L/min and 90.4 ± 3.9%) ($p = 0.003$ and $p < 0.0001$, respectively, Fig. 2A). As expected, acute hypoxia produced an increase in ventilation in both groups; however, the level of $\dot{V}_I$ for pre-menopausal Tibetan women was significantly higher (16.8 ± 3.4 L/min) than in Tibetan post-menopausal women (14.2 ± 2.8 L/min, $p < 0.0001$, Fig 2A), resulting in a significantly higher SpO_2 during hypoxia for pre-menopausal Tibetan women (80.5 ± 3.8%) compared to post-menopausal women (77.2 ± 4.6%, $p < 0.0001$, Fig. 2A). We quantified the HVR and found that pre-menopausal Tibetan women have significantly higher isocapnic HVR values (0.34 ± 0.24) compared to those obtained for post-menopausal women (0.21 ± 0.16, $p < 0.0001$, Fig. 2B). We further explored if these differences are due to changes in V_T or in f_R. We found that pre-menopausal Tibetan women had higher values of V_T breathing room air (0.68 ± 0.13 L) and during acute hypoxia (0.84 ± 0.17 L) compared with post-menopausal women (0.61 ± 0.10 L, $p = 0.0002$ for room air; and 0.71 ± 0.14 L, $p < 0.0001$ for hypoxia, Fig. 2C) at high altitude. However, we did not find significant differences in the values of R_f when we compared post-menopausal and pre-menopausal women (Fig. 2D). We did not find significant differences in the levels of $P_{ET}CO_2$ between groups while breathing room air (30.91 ± 2.17 mmHg for pre-menopausal; and 31.44 ± 2.64 mmHg for post-menopausal women, $p = 0.13$).

Table 2.2: Physiological parameters during room air and acute hypoxia conditions. Mean ± SD.

Parameter	**Tibetan Women**		**t-test**
	Room Air (n)	Acute Hypoxia (n)	***P***
Inspired O_2 (%)	13.5 ± 0.23 (366)	9.2 ± 0.64 (366)	****
SpO_2, (%)	91.0 ± 3.87 (366)	77.8 ± 4.66 (366)	****
$\dot{V}_I$ (L/min)	11.72 ± 1.80 (366)	14.69 ± 3.06 (366)	****
V_T (L)	0.624 ± 0.104 (366)	0.732 ± 0.154 (366)	****
R_f (BrPM)	19 ± 3.3 (366)	20 ± 3.5 (366)	****
$P_{ET}CO_2$ (mmHg)	31.2 ± 2.57 (366)	31.5 ± 2.55 (366)	n.s.
HR (BPM)	72 ± 8.9 (365)	81 ± 9.6 (365)	****

Students' t-test, **** $p < 0.0001$.

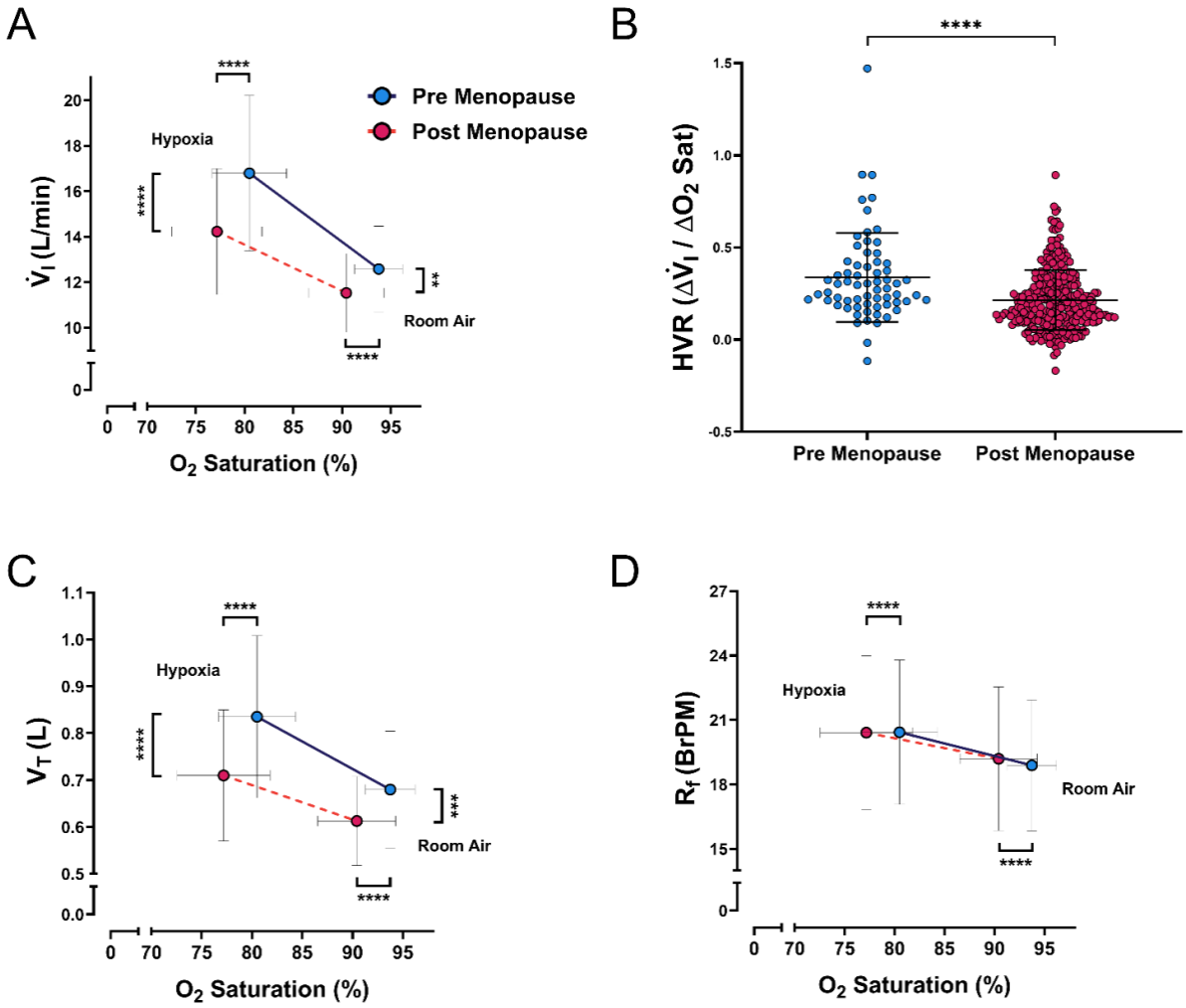


Figure 2.2: Ventilatory responses during room air and isocapnic hypoxia. (A) Both groups increased ventilation when exposed to acute hypoxia compared to room air. The slopes from (A) are the average HVR values depicted in (B), where pre-menopausal women had a significantly higher HVR than post-menopausal women. (C) Pre-menopausal women had a higher V_T than post-menopausal women but (D) R_f did not differ between the two groups.

Hypoxic heart rate response

We did not find differences in HR between pre- and post-menopausal women in room air conditions (71.5 ± 9.0 BPM for post-menopausal versus 72.7 ± 8.4 BPM for pre-menopausal Tibetan women). However, during exposure to hypoxia at high altitude, pre-menopausal Tibetan women have significantly higher values of HR (84.4 ± 8.5 BPM) compared to the post-menopausal group (80.0 ± 9.7 BPM, p = 0.0005, Fig. 3A). The quantification of HHRR reveal that pre-menopausal Tibetan women have significantly higher HHRR (0.911 ± 0.383) compared to the values observed for post-menopausal Tibetan women (0.649 ± 0.278, p < 0.0001, Fig. 3B).

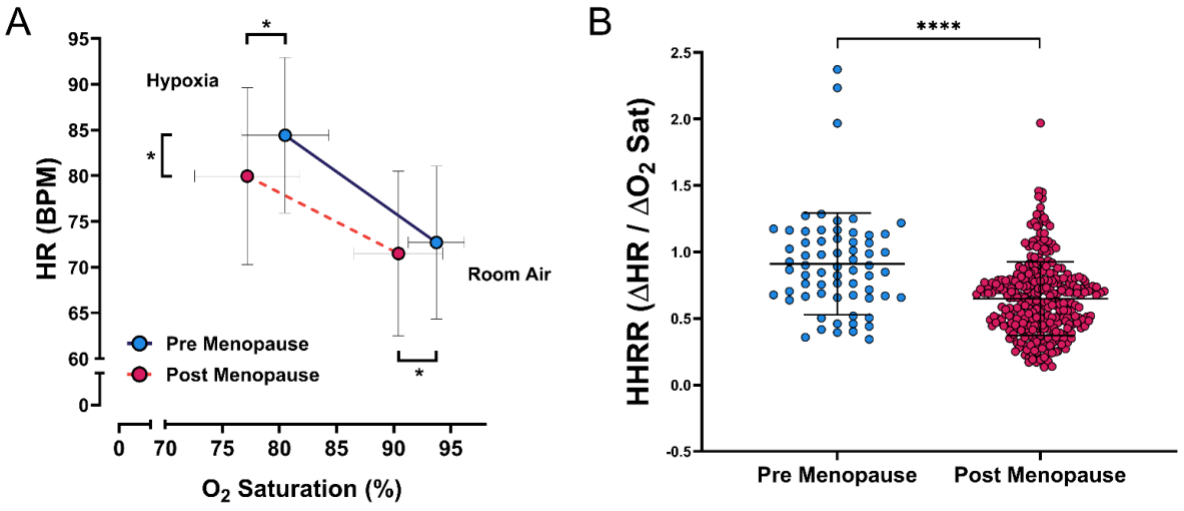


Figure 2.3: Heart rate responses during room air and isocapnic hypoxia. (A) Both groups increased heart rate when exposed to acute hypoxia compared to room air. The slopes from (A) are the average HHRR values depicted in (B), where pre-menopausal women had a significantly higher HHRR than post-menopausal women.

Age is correlated with hypoxic ventilatory and heart rate responses only in post-menopausal women

Because other authors have reported that HVR and HHRR, measured during exercise, change with age (Lhuissier, F. J. et al., 2012; Richalet and Lhuissier, F. J., 2015), it is plausible that the differences in ethnically Tibetan women pre- and post-menopause may be due to age. Therefore, we decided to study the correlations of HVR and HHRR within age ranges, and compared the slopes and intercepts (elevations) resulting from linear regressions for both groups of pre- and post-menopausal Tibetan women. HVR is significantly correlated with age for Tibetan women in post-menopause (p=0.0002) but is not in the pre-menopause group (Fig. 4A). Similarly, post-menopausal show a correlation of HHRR with age ($p < 0.0001$), although this correlation was not observed in the pre-menopausal group (Fig. 4B).

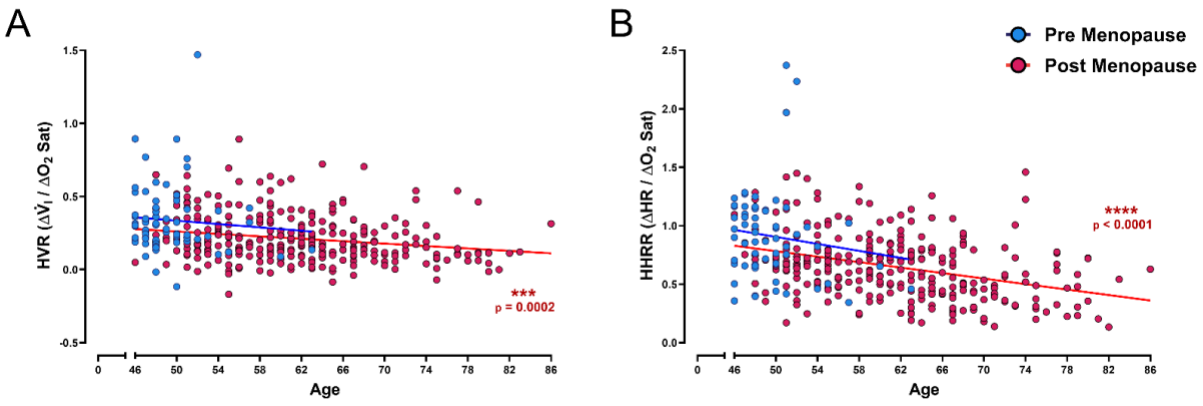


Figure 2.4: HVR and HHRR correlations with age. (A) HVR and (B) HHRR significantly correlated with age in the post-menopausal group but not the pre-menopausal group.

The linear regression analysis did not show significant differences in slope between pre- and post-menopausal women for either HVR or HHRR versus age. However, the comparisons of linear regression intercepts between pre- and post-menopausal Tibetan women show significant

differences for HVR ($p = 0.0087$) and HHRR ($p = 0.0072$) when displayed versus age (Figs. 4A and 4B, red line versus blue line for each graph).

Ventilatory and heart rate responses to hypoxia are correlated with oxygen saturation independent of menstrual status

HVR was positively correlated with room air O_2 saturation for pre-menopausal ($p < 0.001$) and post-menopausal Tibetan women ($p < 0.0001$, Fig.5A). Analysis of linear regressions of HVR versus room air O_2 saturations revealed no significant differences on the slopes of pre- and post-menopausal Tibetan women ($p = 0.086$); however, the intercepts were significantly different between pre-menopausal and post-menopausal Tibetan women ($p < 0.0001$, Fig. 5A, red versus blue lines).

Similarly, we found positive correlations of HHRR with room air O_2 for Tibetan women in pre- ($p < 0.001$) and post- ($p < 0,0001$, Fig. 5B) menopausal stages. The analysis of differences in the linear regression for both groups revealed that the slopes of the groups were not significantly different ($p = 0.1226$), although the intercepts of pre- and post-menopausal Tibetan women were significantly different ($p < 0.0001$, Fig. 5B, red versus blue lines).

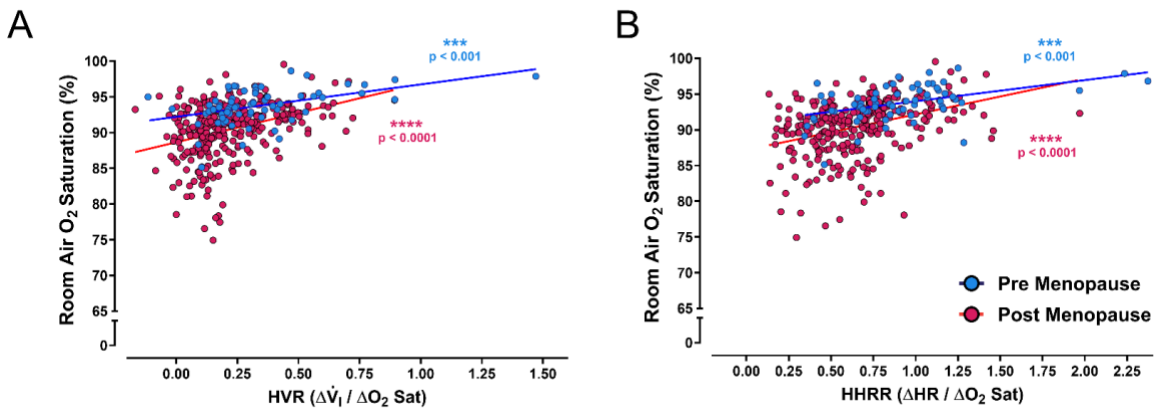


Figure 2.5: HVR and HHRR correlations with oxygen saturation in room air. (A) HVR and (B) HHRR significantly correlated with room air SpO_2 in both the post-menopausal group and the pre-menopausal group.

HVR and HHRR are positively correlated in pre- and post-menopausal women

To study coordinated ventilatory and heart rate responses to hypoxia in Tibetans women at high altitude and the effects or menopausal status, we explored the associations between HVR and HHRR in our pre- and post-menopause groups. We found a positive significant correlation between HVR and HHRR for both pre-menopause and post-menopause groups ($p < 0.0001$ for both) of Tibetan women living at high altitude (Fig. 6). The linear regression analysis did not show significant differences between the slopes of the groups, although their intercepts are significantly different ($p < 0.0001$, blue versus red line, Fig. 6).

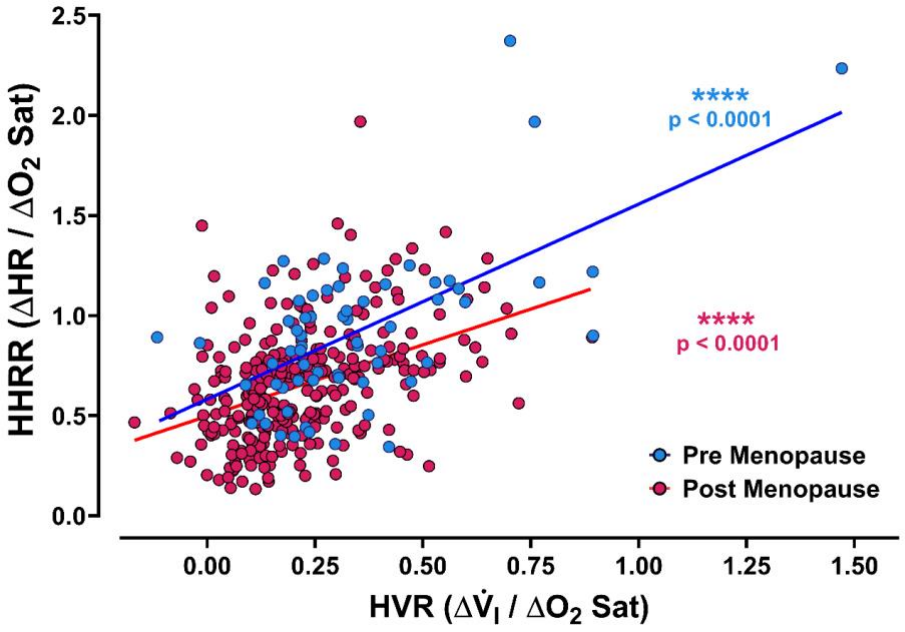


Figure 2.6: HVR and HHRR are significantly correlated in both groups.

Genome-wide association study

To assess whether the biological variables related to HVR are associated with genetic variation, we performed a GWAS. $P_{ET}CO_2$ in room air was associated with 42 SNPs (Table 3) and $\dot{V}_I$ in hypoxia was associated with one single-nucleotide polymorphism (SNP) (Table 3) at genome-wide significance ($p < 5 \times 10^{-8}$). The genetic region associated with $P_{ET}CO_2$ in room air is located in Chromosome 1 and spans approximately 80 kb. This region does not overlap with any known genes and the closest, potentially functional genetic component, encodes for the pseudogene Cyclin Dependent Kinase 4 Pseudogene 1 (*CDK4P1*) (Kent et al., 2002) approximately 23 kb away. The single SNP associated with $\dot{V}_I$ in hypoxia is located within an intronic region of calsyntenin 2 (*CLSTN2*).

Table 2.3: Genome-wide association study results testing for genetic associations with ventilatory measurements.

Trait	CHR	Number of SNPs	Rs ID of top SNP	Minor allele frequency	β	Std Error of β	P_LRT
$P_{ET}CO_2$ in room air	1	42	rs55865785	0.316	1.31 mmHg	0.270	$< 5 \times 10^{-8}$
$\dot{V}_I$ in hypoxia	3	1	rs34947371	0.314	1.32 L/min	0.316	4×10^{-8}

Positive selection scan

To assess whether regions of the genome under positive selection are associated with control of breathing, we identified SNPs under positive selection using iSAFE (Akbari et al., 2018) and overlapped the genes within 100 kb of these SNPs with a control of breathing candidate gene list (Supplementary Table 1). The results of this overlap are listed in Table 4. The positively-selected SNP rs712824 is a strong expression quantitative trait locus (eQTL) for coiled-coil-helix-coiled-coil-helix domain containing 2 (*CHCHD2*) in various tissues (GTEx Consortium, 2013).

Table 2.4: Results of positive selection scan results overlapped with control of breathing candidate gene list.

Gene	iSAFE Score	Physiological Outcome of Interest for High Altitude	Ref.
EPAS1	0.617	**HIF-2*α*.** Involved in hypoxic ventilatory response and carotid body morphology.	Moreno-Domínguez, et al. 2020; Ivy et al, 2022
PPARG	0.534	**PPARγ.** Associated with obstructive sleep apnea in Han Chinese and Asian Indians. Under positive selection in high-altitude Mongolians.	Xing et al. 2013; Liu et al. 2010; Jiao et al. 2019; Bhushan et al. 2011
CHCHD2	0.419	**Eukaryotic CX(9)C protein.** Upstream regulator of COX4I2, mitochondrial cytochrome subunit crucial for hypoxic ventilatory response.	Aras et al. 2013; Moreno-Domínguez, et al. 2020
EGLN1	0.308	**PHD2.** Regulator of HIF activity.	Simonson et al 2010; Jeong et al. 2018; Petousi et al. 2014

Discussion

This is the first study demonstrating an association between menopausal status with physiological (ventilatory and heart rate) responses to acute hypoxia in a large cohort of Tibetan women residing at high altitude. Our results show that, compared to post-menopausal women, pre-menopausal Tibetan women have higher minute ventilation during room air and acute hypoxic conditions, elevated HVR, higher values of HR during hypoxia, and increased HHRR. HVR and HHRR correlated negatively with age in the post-menopausal Tibetan women but not the pre-menopausal, both HVR and HHRR positively correlated with O_2 saturation (independent of menopausal status), and HVR and HHR positively correlated between one another in both pre- and

post-menopausal groups. We identified 42 SNPs associated with $P_{ET}CO_2$ in room air and one SNP associated with $\dot{V}_I$ in hypoxia at genome-wide significance within this Tibetan cohort. We further identified four genes (*EPAS1*, *EGLN1*, *PPARG*, and *CHCHD2*) that may play roles in control of breathing to be under positive selection within the same cohort.

Ventilatory and cardiac responses to hypoxia

Several studies explore the sex differences in ventilatory control (Behan and Kinkead, 2011; Gargaglioni et al., 2019), identifying elevated HVR for women compared to men after adjusting for body surface area (Aitken et al., 1986), women with higher resting ventilation than men depending on the phase of menstrual cycle (Macnutt et al., 2012), and effects of progesterone and estrogen on ventilation (LoMauro and Aliverti, 2021). However, others report lower HVR in female versus males even after body-surface-area correction (Goldberg et al., 2017). This topic is still a matter of debate, with the possibility of different effects due to the impact of the sexual hormones (and the menstrual cycle) on ventilation, the dose of hormonal treatment, and the species used in each given study (Gargaglioni et al., 2019; Raberin et al., 2023). These differences highlight the necessity for more studies exploring the effects of sex, menstrual, or hormonal status on the physiological consequences of hypoxia.

Progesterone increases ventilation, as demonstrated in humans studies in males supplemented with progesterone (Okita et al., 1987; Zwillich et al., 1978) and in studies performed in postmenopausal women with hormone therapy (Cistulli et al., 1994). Animal studies confirm the excitatory role of progesterone, since experiments performed in cats show that administration of progesterone increases phrenic nerve activity through a central-driven mechanism (Bayliss et al., 1987), and that ovariectomized cats show reduced HVR without changes in resting ventilation

(Tatsumi et al., 1997). Estrogen also contributes to increased ventilatory responses, showing a synergistic effect with progesterone, and potential effects directly in centers of the brainstem that control ventilation (Behan and Wenninger, 2008; Behan et al., 2003; Cistulli et al., 1994). Ovariectomized rats with low levels of estradiol show diminished magnitude of phrenic long-term facilitation after acute intermittent hypoxia two weeks after surgery, without changes in the values of baseline ventilation (Barok et al., 2021), showing the possibility of different mechanisms for different components of ventilatory responses to hypoxia. Considering these effects, it is expected to find differences in ventilatory responses to hypoxia with the phases of the menstrual cycle. Previous studies show increased resting ventilation and Studies comparing ventilatory traits of Tibetan women to others (with and without a history of high-altitude residence) may help to elucidate if the observed magnitude of change, pre- and post-menopause, are unique to Tibetan women or universal. These comparisons are difficult due to the different techniques and protocols used to measure ventilatory responses to hypoxia at high altitude. In reference to other studies focused on ventilatory responses on women at high altitude, Tibetan women in this study had higher resting $\dot{V}_I$ and higher O_2 saturation compared with sea-level residence women traveling to high altitude (Muza et al., 2001). Leon-Velarde et al. (2001) studied Andean pre- and post-menopausal women residing at Cerro de Pasco, Perú for more than 10 years, reporting higher values of O_2 saturation and lower values of $P_{ET}CO_2$ in their pre- versus post-menopausal groups in an effect attributed to progesterone. Although, their overall values of O_2 saturation are lower and $P_{ET}CO_2$ higher than those observed in our Tibetan cohort (Fig. 2 and $P_{ET}CO_2$ mentioned in results), suggesting that Tibetan women (pre- and post-menopausal) have higher ventilation that their Andean counterparts (León-Velarde et al., 2001). This agrees with comparisons between Tibetan and Andean women without considering their menopausal status (Beall, 2007).

The values of resting ventilation and HVR that we observed are lower than those reported before for Tibetan women (Beall et al., 1997), although the female group studied in the mentioned publication considered adult women starting at 20 years old. It is then plausible that differences in age may explain the lower values that we observed in our study (Fig. 2 and discussed below). Interestingly, the levels of $\dot{V}_I$ while breathing room air in post-menopausal women in this study are similar to the levels observed in male Tibetan residents of high altitude, and both are higher than the resting $\dot{V}_I$ levels observed in male Han Chinese at high altitude (Curran et al., 1997), suggesting the possibility of increased ventilation acting as an adaptive feature in Tibetan pre-menopausal women.

Regarding measurements of HR at high altitude, our results did not show significant differences while breathing room air. Our recorded values are comparable to those observed for Tibetan women breathing room air at high altitude before (Beall et al., 1997), and to Andean women that did not show significant differences in pulse rate due to menopausal status while breathing room air at high altitude (León-Velarde et al., 2001). The HR values that we reported while breathing room air are slightly higher than those observed for male and female Tibetans while breathing hyperoxic gas at intermediate altitude, although the increased HR with hypoxic isocapnic stimulus is similar (Moya et al., 2024). Studying responses to acute hypoxia, Richalet et al. (2020) found that a measurement of hypoxic heart rate response, but during exercise, was decreased in post-menopausal women. The authors attribute this effect to age differences more than menopausal status, because the differences that they observed in HR were not modified with hormonal treatment. Considering that the protocols used were different, these results agree with our finding of elevated HR during hypoxia and HHRR values for pre-menopausal Tibetan women compared to the post-menopausal Tibetan group (Fig. 3).

Relationship of HVR, HHRR, age, and baseline O_2 saturation

We found a significant negative correlation of HVR and HHRR with age in the post-menopausal but not in the pre-menopausal group of Tibetan women. The effects of age on ventilatory and cardiovascular responses to high altitude hypoxia has been studied before (Beall et al., 1997; Lhuissier, F. J. et al., 2012; Richalet et al., 2023; Richalet and Lhuissier, F. J., 2015; Richalet et al., 2020). Lhuissier et al. (2021) did not find effects of age on HVR during resting conditions in French women traveling to high altitude, and Beall et al. (1997) did not find significant correlations between HVR and age in a group of Tibetan women starting from 20 years and older. These results agree with our finding on absent correlation of HVR and age in pre-menopausal Tibetan women (Fig. 4A). Lhuissier et al. (2012) found a progressive decrease of resting HHRR after 40 years old in untrained European women similar to data observed in men, but not in trained women. Interestingly, HVR and HHRR during exercise was significantly affected by menopausal status but without effects of hormone treatment, highlighting the possibility of different mechanisms triggered by HVR and HHRR alone, versus during exercise (Richalet and Lhuissier, F. J., 2015; Richalet et al., 2020).

Our results showing correlations of HVR and HHRR with age only in post-menopausal Tibetan women allow the possibility of a hormone-driven mechanism occurring only in the Tibetan population that may provide an adaptive benefit to high-altitude hypoxia in pre-menopausal women. The potential benefit would be the direct increase of room air O_2 saturation with higher HVR and HHRR, independent of menopausal status (Fig. 5). Beall et al. (1997) did not find correlation of HVR with O_2 saturation, although she did find a correlation between resting ventilation and O_2 saturation.

In order to understand a coordinated hypoxic response, we correlated the HVR and the HHRR. Richalet and Lhuissier (2015) have elegantly suggested that the ventilatory and heart rate responses to hypoxia during exercise may be opposite, complementary, and changing with age, but ensuring the proper levels of O_2 saturation across lifetime. On the contrary, we found a positive correlation between HVR and HHRR (without exercise) in both pre- and post-menopausal women (Fig. 6), and we have shown a positive correlation of these two factors in poikilocapnic hypoxia for grouped Tibetan male and females living at intermediate altitude, but not for Han Chinese (Moya et al., 2024). Differently to what occurs during exercise in populations living at sea level, it is possible that Tibetans may have and adaptive coordinated physiological response as a consequence of exposure to hypoxia for generations (Yu, J. J. et al., 2022), this may result in an additive contribution of higher values of HVR and HHRR to ensure oxygen transport, as it may occur by other mechanisms in deer mice exposed to high altitude for generations (Ivy and Scott, 2017; Ivy et al., 2022). Indeed, Zhou et al. (2008) studied Tibetans born and raised at high altitude (3700m) who migrated to sea level for four years and compared them with a lowland Han Chinese group. The authors exposed both groups to acute hypoxia in a hypobaric chamber (2 hours, simulating 3700m) and found that acute hypoxia induced a significant increase of heart rate in the Han Chinese but not in the Tibetan population. Although this study was performed only in men, it suggests a permanent effect of ancestry history on physiological responses to hypoxia.

Genome-wide association study

We identified 42 SNPs that associated with $P_{ET}CO_2$ in room air (with a β ranging between 1.22 and 1.31 mmHg) and one SNP that associated with $\dot{V}_I$ in hypoxia (β equal to 1.32 L/min) at the genome-wide significance level (Table 3). To the best of the authors' knowledge, this is the

first GWAS conducted in a high-altitude population specifically seeking genetic associations with ventilatory measurements. One potential reason why a similar study hasn't been conducted before may be due to collecting enough ventilatory measurements, which can be logistically difficult in the field, to obtain the statistical power required for an analysis such as the GWAS. All 42 SNPs that associated with $P_{ET}CO_2$ in room air were co-located on chromosome one and the closest, potentially functional genetic component is 23 kb away and encodes for the pseudogene *CDK4P1*. Though pseudogenes have historically been thought of as "junk DNA", increasing numbers of studies have shown that pseudogenes may have functional effects on their coding genes (Pink et al., 2011). In this case, *CDK4P1* is a pseudogene for Cyclin Dependent Kinase 4 (*CDK4*), a key player in cell cycle control and tumorigenesis (Baker and Reddy, 2012). The single SNP associated with $\dot{V}_I$ in hypoxia is located within an intronic region of *CLSTN2*, which contributes to neuronal development and synaptic organization (Lipina et al., 2016) and is highly connected to a module of genes that are differentially regulated throughout development in highland deer mice (Schweizer et al., 2023).

Positive selection scan overlapped with candidate gene list

We identified four genes that exhibit evidence of positive selection and have been previously implicated in playing a role in control of breathing (Table 4). Endothelial PAS domain-containing protein 1 (*EPAS1*, which encodes hypoxia-inducible factor 2-alpha, *HIF-2A*) and Egl-9 Family Hypoxia Inducible Factor 1 (*EGLN1*, which encodes prolyl hydroxylase domain 2, *PHD2*) have repeatedly been identified as targets of natural selection in high-altitude populations (Bigham et al., 2010; Gray, O. A. et al., 2022; Jeong et al., 2018; Jorgensen et al., 2023; Lawrence et al., 2024; Lorenzo et al., 2014; Simonson et al., 2010; Song, D. et al., 2020; Xu, S. et al., 2011;

Yi, X. et al., 2010; Yu, J. J. et al., 2022). *EPAS1*/*HIF2A* in particular has been shown to affect the HVR in mice (Hodson et al., 2016; Moreno-Domínguez et al., 2020) and ventilatory sensitivity and carotid body growth in high-altitude deer mice (Ivy et al., 2022). Studies in mice show that *EGLN1* is involved in control of breathing, as demonstrated by increased HVR with knockout deletion on prolyl hydroxylase 2 (Bishop et al., 2013; Hodson et al., 2016). In addition, EGLN1 was previously identified to be associated with higher aerobic capacity (VO_2max) in hypoxia in Andean highlanders (Brutsaert, T. D. et al., 2019), although it's unclear what physiological traits contributed to this increased VO_2max. Peroxisome proliferator-activated receptor gamma (*PPARG*), known to play a role in insulin sensitivity and glucose metabolism (Bhushan et al., 2011), was previously identified as a candidate gene under positive selection in high-altitude Mongolians (Xing et al., 2013) and Tajiks (Liu, X.-L. et al., 2010), and also associated with obstructive sleep apnea in Han Chinese (Jiao et al., 2019) and Asian Indians (Bhushan et al., 2011). Coiled-coil-helix-coiled-coil-helix domain containing 2 (*CHCHD2*) encodes for the protein CHCHD2, which is an upstream regulator of cytochrome c oxidase subunit 4I2 (*COX4I2*) (Aras et al., 2013). Moreno- Domínguez et al. (2020) showed that *COX4I2*-null mice had blunted HVRs. The iSAFE analysis reported a SNP (rs712824) as driving the positive selection signal at the *CHCHD2* locus, and this SNP is a strong expression quantitative trait locus (eQTL) for CHCHD2 in various tissues (GTEx Consortium, 2013). To the best of the authors' knowledge, this is the first report of *CHCHD2* showing evidence of being under positive selection.

Conclusion

The study of high-altitude populations, as Tibetans, provide a powerful insight into individual variation in the responses to hypoxia. Control of breathing have been hypothesized as

one of the main features of adaptation observed in high altitude populations, however this is the first study exploring the contribution of pre- and post-menopause status on the ventilatory and heart rate responses to acute hypoxia in Tibetan women living at high altitude. We reported that pre-menopausal women have higher ventilatory and heart rate responses to acute hypoxia. However, more studies comparing these responses with those observed in women and men from populations living at sea level need to be performed. It is plausible that the increased physiological responses observed in Tibetan pre-menopausal women contributes to their adaptation to high-altitude conditions. We report the first genome-wide association and analysis of adaptive candidate genes prioritized through powerful test of selection in Tibetan women living at high altitude. We provide promising candidates and insight into potential genetic contributors to control of breathing in highlanders. Our experiments contribute with valuable knowledge on control of breathing with potential applications in critical and chronic diseases involving hypoxia.

Acknowledgements

Chapter 2, in full, is currently being prepared for submission for publication of the material. Moya, Esteban A.; Yu, James J.; Gu, Wanjun; Di Rienzo, Anna; Witonsky, David; Craig, Sienna R.; Powell, Frank L.; Beall, Cynthia M.; Simonson, Tatum S. The dissertation author was co-first author of this paper.

Supplemental Table 2.1: Control of breathing candidate gene list.

Gene	**Reference**	**Summary**
COX4i2	(Moreno-Domínguez et al., 2020)	Protein coding gene involved in respiratory electron transport. Knockout results in similar phenotype to EPAS1 knockout in mice.
COX4i1	(Hwang, H. J. et al., 2015)	May also be involved in respiratory electron transport chain
EPAS1	(Moreno-Domínguez et al., 2020)	Involved in HIF pathway
EGLN1	(Brutsaert, T. D. et al., 2019)	Involved in HIF pathway
PHOX2B	(Dubreuil et al., 2009)	Associated with breathing disorders
MECP2	(Mackay et al., 2017)	Associated with Rett Syndrome
EGR2	(Chatonnet et al., 2007)	Associated with breathing abnormality
MAFB	(Blanchi et al., 2003)	Knockout mice die from central apnea at birth
BDNF	(Balkowiec and Katz, 1998)	Required for normal respiratory rhythm in mice
GAD1	(Kuwana et al., 2003)	Knockout disrupts respiratory control in mice
ASCL1	(Dauger et al., 1999)	Affects ventilatory responses to hypercapnia and hypoxia in mice
TLX3	(Qian et al., 2001)	Rnx-deficient mice die from respiratory failure
NR4A2	(Dong et al., 2016)	In Nurr1-ablated DA neurons, more than 90% of genes encoding respiratory chains are downregulated
ADCYAP1	(Peña, 2010)	Involved in breathing control and rhythm
HMOX1	(Ayer et al., 2016)	Involved in cardiovascular diseases in hypoxia context
HMOX2	(Ayer et al., 2016)	Varying levels of hypoxic sensitivity of the carotid body in rat strains
PPARG	(Jiao et al., 2019)	Associated with sleep apnea in Han Chinese
CHCHD2	(Aras et al., 2013)	Upstream of COX4I2
OR51E2	(Chang et al., 2015)	Olfr78 mutants fail to increase ventilation in hypoxia but respond normally to hypercapnia. May act as a hypoxia sensor
EGLN3	(Gao, L. et al., 2017)	Differentially expressed in the carotid body compared to the superior cervical ganglion during hypoxia in adult mice
ARNT2	(Gao, L. et al., 2017)	Differentially expressed in the carotid body compared to the superior cervical ganglion during hypoxia in adult mice
VEGFA	(Gao, L. et al., 2017)	Differentially expressed in the carotid body compared to the superior cervical ganglion during hypoxia in adult mice
VEGFC	(Gao, L. et al., 2017)	Differentially expressed in the carotid body compared to the superior cervical ganglion during hypoxia in adult mice
NOTCH1	Preliminary unpublished data	Associated with ventilatory phenotypes
PRKAA1	Preliminary unpublished data	Associated with ventilatory phenotypes

Chapter 3 Bacteriotherapy associated with elevated oxygen saturation and improved acclimatization at high altitude

Abstract

Background:

High altitude imposes significant physiological stress on the human body due to reduced oxygen availability. Aids to improve acclimatization are limited, and specific bacteriotherapy offers a potential avenue to improve acclimatization. SLAB51, a multi-strain probiotic, improves oxygen saturation and could benefit individuals traveling to high altitudes.

Methods:

Participants (n = 17) underwent a randomized, doubled-blinded, placebo-controlled study to test the effects of SLAB51 on acclimatization to high altitude. Measurements were collected at sea level and during a course of up to four days at high altitude (3,800 m). The primary outcome was oxygen saturation (SpO_2) during both daytime and nighttime. Secondary measurements included Acute Mountain Sickness (AMS) score, sleep measurements, ventilation, resting heart rate, blood pressure, heart rate variability, and fasting glucose levels.

Findings:

The SLAB51 group exhibited a higher daytime SpO_2 (mean ± SD; 90.2 ± 2.8 %) compared to the placebo group (86.7 ± 4.4 %) with a mean difference of 3.6% ($p < 0.0001$, 95% CI [2.1, 5.0]) and a higher average nighttime SpO_2 (81.9 ± 4.1 %) compared to the placebo group (76.9 ± 6.6 %) with a mean difference of 5.1% ($p = 0.011$, 95% CI [1.2, 9.0]). A mixed-effects model predicting daytime SpO_2 and considering hypoxic ventilatory response, age, sex, and body-mass index further supported a higher SpO_2 in the probiotic group (estimate = 2.5%, t-value = 2.31). The probiotic group also exhibited a lower AMS score (2.3 ± 1.8) than the placebo group (4.9 ±

2.4) during the first two days at high altitude with a mean decrease of 2.5 (p = 0.0026, 95% CI [-4.1, -0.9]).

Interpretation:

SLAB51 ingestion enhances acclimatization to high altitude, evidenced by higher SpO_2 levels and lower AMS scores in treatment versus placebo groups. These results suggest specific bacteriotherapy as a novel, non-invasive intervention for high-altitude acclimatization.

Funding:

LEA Altitude Performance Fund

Introduction

The reduced availability of oxygen (O_2) at high altitude imposes various physiological stresses on humans, including decreased O_2 saturation, disturbed sleep (Ainslie et al., 2013; Reite et al., 1975), increased sympathetic activation (Simpson et al., 2024), reduced exercise capacity (Sutton, J. R. et al., 1983), and symptoms of acute mountain sickness (AMS), e.g., fatigue, headache, and shortness of breath (Imray et al., 2010; Singh, I. et al., 1969). Despite these negative effects, millions of people ascend to high altitude every year for work and recreation (Zafren and Honigman, 1997), and many of these individuals become incapacitated or experience debilitating AMS symptoms (Imray et al., 2010). In extreme cases, some individuals develop life-threatening conditions, such as high-altitude pulmonary edema (HAPE) and high-altitude cerebral edema (HACE) (Basnyat and Murdoch, 2003). Aside from slow ascent to altitude (Hackett and Roach, R. C., 2001), limited aids are available to improve acclimatization, require a prescription, and/or have side-effects. Therefore, non-invasive interventions to aid in high-altitude acclimatization would be of great benefit.

The human body harbors internal and external microorganisms. In particular, the gut hosts bacteria, archaea, and eukarya in the gastrointestinal tract, collectively known as the gut microbiota. These organisms play a critical role in health and disease (Thursby and Juge, 2017). Given that a large proportion of cardiac output is directed towards the intestines to transport O_2 and other nutrients (Granger et al., 2015), modulation of the human-gut microbiota interface may be an avenue to improve systemic oxygenation. SLAB51 is a blend of eight different live proprietary bacterial strains: *Streptococcus thermophilus*, two strains of *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Lactobacillus brevis* (ExeGi Pharma LLC, USA). When the probiotic bacteria present in SLAB51 interact with human intestinal epithelial cells, they increase stabilization of hypoxia inducible factor 1 subunit alpha (HIF-1α), a critical regulator of O_2 homeostasis, potentially driving the intestine to utilize O_2-independent pathways of metabolism and reducing O_2 demand (Lombardi et al., 2023). Up to now, SLAB51 has demonstrated promise in a wide range of health conditions such as Parkinson's and Alzheimer's Diseases (Bonfili et al., 2018; Bonfili et al., 2020; Bonfili et al., 2022; Bonfili et al., 2021; Castelli et al., 2020) as well as conditions directly related to O_2 homeostasis such as COVID-19 (Ceccarelli et al., 2021; Ettorre, d' et al., 2020; Trinchieri et al., 2022), preterm birth (Baldassarre et al., 2023), and oxidative stress from sleep loss (Zheng, Y. et al., 2023). Given its value in improving O_2 saturation, SLAB51 has the potential to improve acclimatization to high altitude.

In this study, we investigate the effects of SLAB51 ingestion on acclimatization to high altitude in sea-level residents transported to 3,800 m (12,470 ft). Seventeen participants underwent a randomized, placebo-controlled, double-blinded interventional study: nine individuals ingested the probiotic throughout their stay at high altitude and eight individuals ingested a placebo.

Participants underwent measurements at sea level and stayed up to four days and three nights at high altitude. O_2 saturation, measured via pulse oximetry (SpO_2), during both daytime and nighttime was the primary measurement for intervention efficacy and secondary measurements included AMS score, apnea-hypopnea index (AHI), O_2 desaturation index (ODI), hypoxic ventilatory response (HVR), hypercapnic ventilatory response (HCVR), hypercapnic HVR, hypoxic heart rate response (HHRR), resting heart rate (HR), blood pressure, heart rate variability (HRV), and fasting glucose levels. We hypothesized that individuals who received the probiotic would have a higher SpO_2 during the daytime and nighttime compared to those who received the placebo, thereby improving high-altitude acclimatization.

Methods

Study design

We conducted a randomized, double-blinded, placebo-controlled study to test the effects of the probiotic SLAB51 on acclimatization to high altitude. We collected measurements from participants at sea level in a human laboratory space at the University of California, San Diego (UCSD) and at high altitude at the University of California White Mountain Research Center's (WMRC) Barcroft Station (3,800 m) during a stay of up to four days (three nights). All non-sleep measurements were conducted in laboratory spaces at UCSD or at Barcroft Station. Sleep measurements at sea level were collected in participants' homes and sleep measurements at high altitude were collected at Barcroft Station. Participants were transported via automobile during an approximately eight-hour drive from UCSD to Barcroft Station. Participants consumed either the probiotic or placebo upon arrival at Barcroft Station and up to three times a day during the duration

of their stay. The study was approved by the UCSD Institutional Review Board (Protocol #807971).

Study participants

We recruited self-identified Latino individuals, the second largest "racial or ethnic group" in San Diego County, from the San Diego metropolitan and surrounding areas for this study. Participants were recruited via word-of-mouth, flyers posted throughout the greater San Diego metropolitan area, and emails sent to various Latin American associations and other community groups. Participants were considered eligible if they identified as being Latino, were at least 18 years old and less than 66 years old, and in good general health. Participants were deemed ineligible if they had a history of cardiovascular or pulmonary disease, currently smoked or used tobacco (more than one cigarette a day), had a body mass index (BMI) greater than 35, and/or a STOP-BANG score for obstructive sleep apnea equal or greater than three (https://www.mdcalc.com/calc/3992/stop-bang-score-obstructive-sleep-apnea). Individuals were also excluded for any use of medications or agents that might interfere with physiological measurements, if they travelled above 2,500 m (8,202 feet) of elevation during the four weeks prior to the initial baseline measurements at sea level and throughout the duration of the study. Eligibility was first determined by an online eligibility form and confirmed after physiological measurements were taken in a UCSD laboratory space. Gender was self-reported as: man, woman, genderqueer/non-binary, and prefer not to disclose. Participants provided written informed consent prior to any measurements taken at UCSD.

Randomization and masking

Prior to the high-altitude portion of the study, participants were assigned evenly to either Treatment Group A or B by the computer randomization program randomizeR (Version 3.0.0). Both researchers and study participants were blinded as to whether Group A or B received the probiotic or placebo. Each dose of the probiotic and placebo was contained in a non-transparent sachet and labeled A or B by an assistant who was not part of the study, and only this individual knew which group received which treatment. Only after all data had been collected and preliminary data analyses comparing measurements between the treatment groups were complete were the researchers unblinded.

Procedures

Probiotic and placebo ingestion and administration

Each sachet, labeled A or B, contained six grams of the bacterial mixture. The placebo consisted of all components present in the probiotic formulation except for the bacteria, which were replaced with additional maltose or other safe food additives. Each mixture was dissolved in 100 mL of water and mixed thoroughly. Participants ingested the mixture in front of a researcher who recorded each ingestion and noted the time of ingestion. Participants ingested the probiotic or placebo within 15 minutes after every meal, three times a day on the full days at high altitude (days two and three at high altitude) and one time a day on the day of arrival and the day of departure (day one and four, respectively). Participants were provided with the same foods at all meals to reduce variable effects of different diets. All probiotic and placebo sachets were stored in refrigerated conditions or in a cooler during transportation (2°C- 8°C) and away from light until consumption. While the product can remain up to one week out of the refrigerator up to a maximum

of 25°C without affecting its quality (Prof De Simone, personal communication), the probiotic and placebo sachets were never out of refrigerated conditions for more than 30 minutes.

Oxygen saturation and heart rate measurements

Daytime SpO_2 and resting HR were measured by a pulse oximeter probe (Radical-7, Masimo Corporation, Irvine, CA) that was secured to the forehead by a headband. All measurements were taken at rest in a seated position. Measurements were collected up to three times a day: once in the evening after arrival at high altitude and at least 90 minutes after consumption of the first dose of probiotic or placebo, three times a day during the second and third day at high altitude (generally in the morning, afternoon, and evening), and once in the morning on the last day at high altitude. Nighttime SpO_2 and digital blood flow were measured by fingertip peripheral arterial tonometry and pulse oximetry using WatchPAT device (Itamar Medical, Caesarea, Israel).

Acute mountain sickness score

Acute mountain sickness severity was assessed by the 2018 Lake Louise AMS survey (Roach, R. C. et al., 2018) to determine severity of participants' AMS symptoms. Participants answered the four questions from the survey on an iPad at sea level during initial measurements and every day at high altitude. The results of the four questions were added up to determine a total AMS score for each day.

Sleep measurements

All sleep data were recorded using WatchPAT technology. WatchPAT is a wrist-worn home sleep apnea testing device equipped with a single-finger probe, an actigraph, and a module to detect central sleep apneas (Central PLUS Module). Respiration was monitored through the Central PLUS Module which contains a built-in 3D accelerometer which measures upper chest wall respiratory movements. This data was compared with the peripheral arterial tone signal recorded through a pneumo-optic plethysmograph device in the finger to derive instances of sleep apnea (Pillar et al., 2020). Respiratory events were detected through a combination of techniques including peripheral arterial tone signal attenuation, HR variations, and pulse oximetry desaturation, analyzed through WatchPAT's proprietary software algorithm (Ayas, N. T. et al., 2003). The AHI was classified based on either a 4% O_2 desaturation or a 3% O_2 desaturation accompanied by tonometry changes, suggestive of arousal, aligning closely with the Chicago criteria. The ODI was assessed through an internal algorithm. Device data underwent automated scoring through the WatchPAT's proprietary algorithm and subsequently underwent scoring by a registered polysomnographic technologist.

Ventilatory and heart rate responses to hypoxia and hypercapnia

Participants completed a control of breathing protocol as used in our previous high-altitude studies (Heinrich et al., 2020). At sea level, participants completed an abbreviated, approximately ten-minute, version of the full control of breathing protocol, which provided an opportunity for them to familiarize with the experimental set up and to establish appropriate gas flows for targeted SpO_2 and end-tidal P_{CO2} ($P_{ET}CO_2$). Participants returned to complete the full protocol at sea level after a >30-minute rest period, which provided sufficient time for recovery from hypoxic

ventilatory decline that could be induced by the abbreviated hypoxia exposure (Easton et al., 1988). At high altitude, participants completed the full protocol on the second day after spending one complete night at high altitude.

During testing, participants sat in a chair and a mask was placed over the mouth and nose (7600 V2 Oro-Nasal Mask, Hans Rudolph, Shawnee, KS). Leaks were checked by asking the participant to inhale against a closed inspiratory valve to ensure a vacuum was produced. The mask was connected to a one-way vented breathing circuit with a non-rebreathing valve (2700 Series, Large, Hans Rudolph). A three-way valve upstream of the mask allowed either room air or gas mixtures of nitrogen, O_2, and carbon dioxide (CO_2) to flow into the circuit. O_2 and CO_2 were continuously sampled right before the mask or non-rebreathing valve directly in front of the mouth, respectively (Model 17620 and 17515A, VacuMed, Ventura, CA, USA). Inspiratory flow was measured by a pneumotachograph (Fleisch No. 3, OEM Medical Inc., Richmond, VA, USA) upstream of the mask and connected to a differential pressure transducer (model CD15, Validyne, Northridge, CA, USA). SpO_2 and HR were measured by a pulse oximeter (Radical-7, Masimo Corporation Irvine, CA, USA) with a surface probe secured on the forehead. All analog signals were processed through a PowerLab 8/30 (ADInstruments, Colorado Springs, CO, USA) and sent digitally to a laptop computer. Raw data were recorded in LabChart 8 (ADInstruments). Gas mixtures were manually controlled with a three-channel rotameter flow meter (Matheson Gas Products, Montgomeryville, PA, USA) that delivered mixtures upstream of the three-way valve at flow rates sufficient to prevent rebreathing.

We continuously measured SpO_2, fraction of inspired O_2 (F_{IO_2}), fraction of inspired CO_2 (F_{ICO_2}), tidal volume (V_T), respiratory frequency (f_R), minute ventilation ($\dot{V}_I$), $P_{ET}CO_2$, and HR during the protocol. Participants breathed ambient air for three to five minutes followed by 10

minutes of mild hyperoxia (PO_2~230 mmHg, simulating 30% F_{IO_2} at sea level). This hyperoxic phase was required to reverse potential hypoxic ventilatory decline in participants, as applied in previous studies with people exposed to continuous hypoxemia at high altitude (Pamenter and Powell, F. L., 2016).

To determine the HVR in isocapnic conditions, participants breathed a normoxic gas mixture during five minutes (PO_2~159 mmHg, simulating a level equivalent of 21% F_{IO_2} at sea level), followed by five minutes of isocapnic hypoxia as we targeted three minutes of stable SpO_2 that produced a decrease of at least 10% from the levels recorded during previous hyperoxic measurements (Heinrich et al., 2020). Isocapnia was achieved throughout the hypoxic phase by manually adding CO_2 to maintain the average $P_{ET}CO_2$ value observed during the last minute of the previous hyperoxic period. Isocapnic hypoxic events with $P_{ET}CO_2$ more than 2 mmHg different than the $P_{ET}CO_2$ observed during the previous normoxic condition within the same participant were removed. Participants were then exposed to five minutes of normoxic hypercapnia by bringing the levels of O_2 to the equivalent of 21% F_{IO_2} at sea level with a 5 mmHg increase in $P_{ET}CO_2$ above the value observed during normoxic isocapnia. The HVR and hypercapnic HVR were calculated as the change in ventilation per decrease in SpO_2 ($\Delta \dot{V}_I$ / ΔSpO_2) under each condition of isocapnic hypoxia and hypercapnia, respectively. The normoxic hypercapnic ventilatory response (HCVR) was calculated as the change in ventilation per increase of five mmHg $P_{ET}CO_2$ ($\Delta \dot{V}_I$ / Δ $P_{ET}CO_2$). The hypercapnic HVR was determined in the final five minutes of the protocol, which examined the difference in $\dot{V}_I$ from the isocapnic, normoxic stimulus to a combined hypoxic and hypercapnic stimulus. In all cases, the average $\dot{V}_I$ was obtained during a minimum 30-second period of the final minute of the normoxic and hypoxic conditions.

Heart rate (HR) was recorded from the pulse oximetry device throughout the entire protocol to measure HR responses to hypoxia, hypercapnia, and the combined hypoxia-hypercapnia stimuli. The average HR was obtained from the same minimum 30-second period as the average $\dot{V}_I$.

Blood pressure measurements

Blood pressure measurements were taken either manually with a manual monitor or by an electric cuff monitor on the participant's right arm while they were in a seated, relaxed position. Three separate measurements were taken during each session and up to three sessions of blood pressure measurements were taken per day at high altitude. Only one session of blood pressure measurements was collected at sea level.

Heart rate variability measurements

Estimation of sympathetic nervous system activity was approached through HR variability analysis of R-R peaks during a ten-minute period of electrocardiogram (lead I) obtained during resting conditions. Recording and analyses were performed at sea level and at high altitude using LabChart (Pro version 8.1.25, ADInstruments, Dunedin, New Zealand) using the default parameters indicated for humans, which includes the standard deviation of average R-R values (300s) and frequency bands of very low frequency (VLF: 0-0.04 Hz), low frequency (LF: 0.04-0.15), and high frequency (HF: 0.15-0.45). From the time-domain analysis, we obtained the standard deviation of R-R intervals (SDRR) and the root mean square value of successive differences between normal heart beats (RMSDD). For frequency domain analysis, we measured power of LF and HF in normal units (nu) and the calculated LF/HF ratio.

Fasting glucose measurements

Fasting glucose measurements were collected by Metene TD-4116 Blood Glucose Monitor at sea level in the morning before departure to Barcroft Station and every morning thereafter at Barcroft Station. Participants were fasting for all measurements.

Outcomes

The primary outcome for this study was O_2 saturation measured via pulse oximetry (SpO_2) during both daytime and nighttime, and secondary measurements included AMS score, sleep parameters AHI and ODI, ventilation, resting HR, blood pressure, control of breathing measurements, and fasting glucose concentration.

Statistical analysis

We hypothesized O_2 saturation would be greater in participants who received the probiotic compared to those who received the placebo. Based off a preliminary, unpublished study that compared SpO_2 in two participants who received SLAB51 versus two participants who received a placebo in an air cabin pressurized to the equivalent of approximately 8,000 feet during commercial flight, a sample size of 17 observations were required for an effect size of 0.8% SpO_2 and 39 observations were required for an effect size of 0.5% SpO_2. Both of these calculations were conducted for a Type I error rate of 5% and power of 80% in XLSTAT version 19.2.2 using the non-central Student distribution with non-centrality parameter. The effects of treatment group (probiotic or placebo) and timepoint on daytime SpO_2 and mean nocturnal SpO_2 were determined by two-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test. Mixed-effects models to predict daytime SpO_2 were completed utilizing the package lme4 (Bates, D. et al., 2015)

(Version 1.1.35.1) in RStudio (Version 2023.6.0.421). To account for the contribution of ventilation to SpO_2, we interchangeably considered HVR on the second day at high altitude, HVR on the third day at high altitude, HVR at sea level, and minute ventilation at room air at high altitude in various models and selected the model with the lowest restricted maximum likelihood (REML) and lowest Akaike information criterion (AIC). We considered the interaction term "Treatment Group * HVR on the second day at high altitude" within the model but dropped the term after it was shown to be an insignificant predictor of SpO_2. Model assumptions were checked with package DHARMa (R package version 0.4.6). The ultimate mixed-effects model predicting daytime SpO_2 included Treatment Group and HVR on the second day at high altitude as covariates with age, sex, and BMI as fixed effects, and participant and timepoint as random effects. The effects of treatment group and timepoint on secondary measurements were determined by two-way ANOVA and with a Bonferroni *post hoc* when appropriate.

Role of the funding source

This study was primarily funded by the LEA Altitude Performance Fund, which provided the probiotic and placebo treatment for the study, but had no role in study design, data collection, data analysis, data interpretation, or writing of the report. Other funding sources include the White Mountain Research Center Minigrant awarded to JJY, the National Defense Science and Engineering Graduate Fellowship awarded to JJY, the National Institutes of Health R01HL145470 awarded to TSS, and AM is funded by NIH, reports income from Eli Lilly, LivaNova, Zoll, and Powell Mansfield, and ResMed provided a philanthropic donation to UCSD.

Results

Study participants

A total of 38 participants were assessed for eligibility for this study between June 29, 2023, and September 10, 2023. Of these 38 individuals, 14 were deemed ineligible, 24 were enrolled, seven decided to not complete the study, and 17 participated (full trial profile in Supplementary Fig. 1). The 17 participants were split up into three separate trips to high altitude between the dates of August 18, 2024, and October 8, 2024. Of these 17 participants, nine spent the full three nights and four days at high altitude while eight spent only one night and two days at high altitude due to inclement weather. Details of the 17 participants' baseline characteristics at sea-level are listed in Table 1.

Table 3.1: Demographic and baseline measurements of study participants at sea level.

Characteristic	**Placebo**, N = 8	**Probiotic**, N = 9
Sex		
Female	3 (38%)	5 (56%)
Male	5 (63%)	4 (44%)
Age (years)	22 (20, 29)	25 (20, 32)
BMI (kg/m^2)	26.5 (23.5, 30.8)	25.1 (23.3, 32.2)
Sea-level SpO2 (%)	99.00 (96.98, 99.23)	98.00 (97.00, 99.00)
Sea-level Resting HR (bpm)	60 (56, 64)	64 (55, 69)
Sea-level AMS Score	0 (0, 0)	0 (0, 1)
Sea-level Mean Nighttime SpO2 (%)	96 (95, 96)	95 (94, 96)
Sea-level AHI 4%	1.4 (0.6, 4.5)	1.2 (0.8, 1.8)
Sea-level ODI 4%	1.7 (1.0, 3.2)	1.1 (0.8, 2.0)
Sea-level Fasted Glucose (mg/dL)	107 (104, 111)	106 (99, 110)
Sea-level Systolic Blood Pressure (mmHg)	128 (115, 133)	114 (110, 130)
Sea-level Diastolic Blood Pressure (mmHg)	85 (78, 90)	70 (67, 73)
Sea-level Mean Arterial Blood Pressure (mmHg)	58 (53, 63)	49 (47, 54)
Sea-Level HVR ($\Delta\dot{V}_I / \Delta SpO_2$)	0.32 (0.12, 0.70)	0.35 (0.29, 0.64)
Sea-Level HCVR ($\Delta\dot{V}_I / \Delta ETCO_2$)	0.53 (0.43, 0.84)	0.78 (0.51, 0.94)
Sea-Level Hypercapnic HVR ($\Delta\dot{V}_I / (\Delta ETCO_2 \times \Delta SpO_2)$)	0.79 (0.51, 1.50)	1.03 (0.45, 1.60)
Sea-Level HHRR ($\Delta HR / \Delta SpO_2$)	0.89 (0.86, 1.42)	1.06 (1.01, 1.71)
Sea-Level SDRR (ms)	66 (43, 100)	61 (42, 75)
Sea-Level RMSSD (ms)	66 (43, 100)	61 (42, 75)
Sea-Level LF Power (nu)	55 (40, 74)	58 (53, 67)
Sea-Level HF Power (nu)	42 (26, 50)	39 (31, 43)
Sea-Level LF/HF	1.33 (0.76, 2.89)	1.47 (1.21, 2.14)

BMI, body mass index; SpO_2, oxygen saturation measured by pulse oximetry; HR, heart rate; AMS, acute mountain sickness; AHI, apnea-hypopnea index; ODI, oxygen desaturation index; HVR, hypoxic ventilatory response; HCVR, hypercapnic ventilatory response; HHRR, hypoxic heart rate response; SDRR, standard deviation of the RR interval; RMSSD, root mean square of successive differences; LF, low-frequency; HF, high-frequency. Data are shown as mean (mean - SD, mean + SD).

Oxygen saturation

All individuals had daytime SpO_2 of at least 96% at sea level (Table 1; Figure 1A) and both groups, individuals who were later given the placebo (n = 8) or probiotic (n = 9), had a reduction in SpO_2 upon ascent to high altitude ($p < 0.0001$ for both groups, paired student's t-test). Compared to the placebo group (mean ± SD; 86.7 ± 4.4 %), the probiotic group (90.2 ± 2.8 %) had a higher daytime SpO_2 at high altitude ($p < 0.0001$, two-way ANOVA with Bonferroni *post hoc*, effect size (ES) = 0.95, 95% CI [2.1, 5.0], Fig. 1A) with a mean increase of 3.6% but no effect of timepoint ($p = 0.47$, two-way ANOVA, Fig. 1A). At sea level, all individuals had mean nocturnal SpO_2 of at least 94% (Table 1). Compared to the placebo group (76.9 ± 6.6 %), the probiotic group (81.9 ± 4.1 %) had a higher mean nocturnal SpO_2 at high altitude ($p = 0.011$, two-way ANOVA with Bonferroni *post hoc*, ES = 0.9, 95% CI [1.2, 9.0], Fig. 1B) with a mean increase of 5.1% but no effect of timepoint ($p = 0.66$, two-way ANOVA, Fig. 1B). A mixed-effects model predicting daytime SpO_2 and considering other potential factors such as HVR, age, sex, and BMI further supported the effect of increased SpO_2 with probiotic ingestion compared to placebo (estimate = 2.5%, standard error = 1.1%, t-value = 2.31, Supplementary Table 1). All mixed-effects models predicting mean nocturnal SpO_2 and considering factors such as HVR, HCVR, hypercapnic HVR, age, sex, and BMI were not significant ($p > 0.05$).

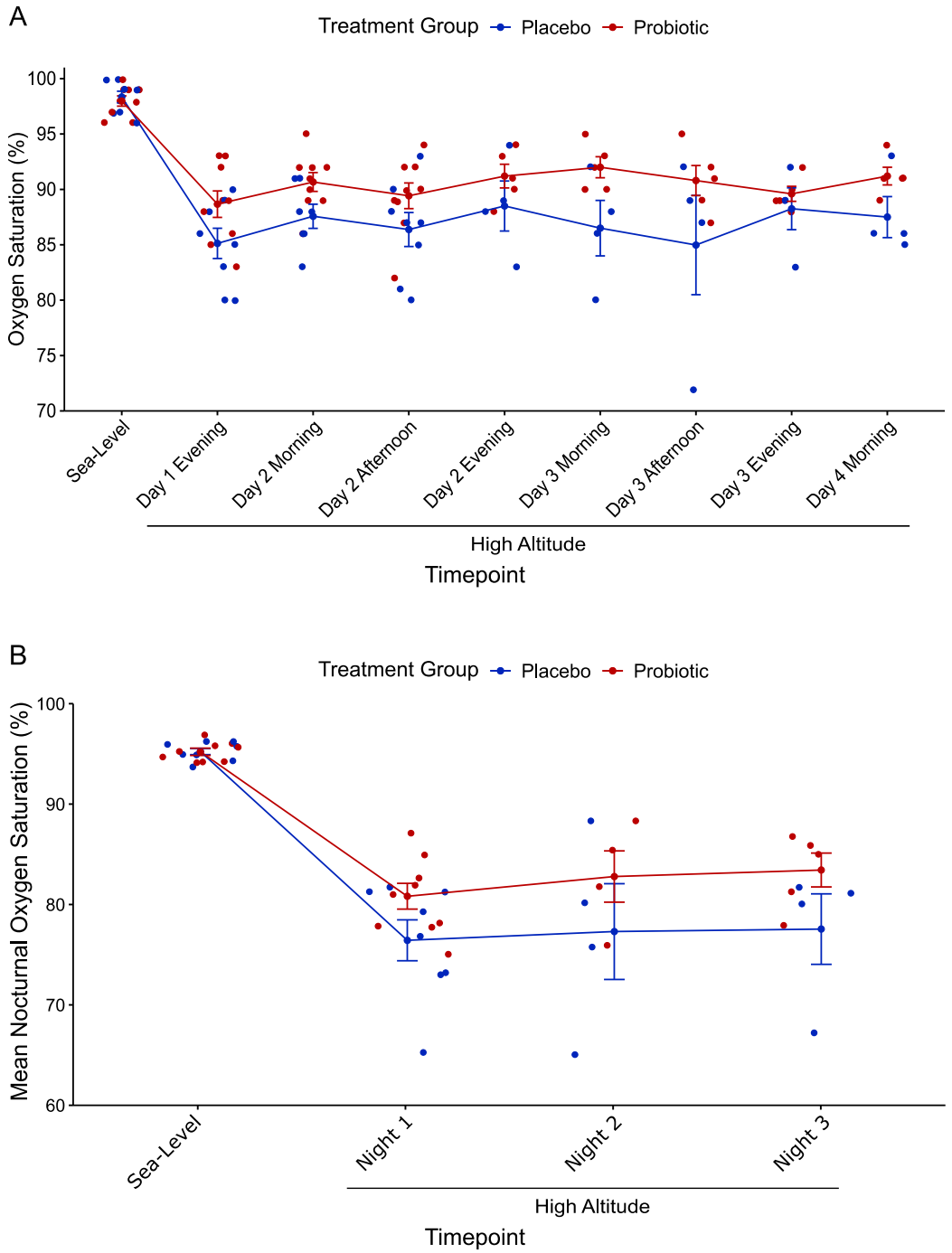


Figure 3.1: Individuals in the probiotic group had increased SpO_2 at high altitude during both daytime and nighttime compared to the placebo group. (A) All individuals experienced a decrease in SpO_2 upon ascent to high altitude ($p < 0.0001$, paired student's t-test). A two-way ANOVA revealed a significant effect of treatment group ($p < 0.0001$) but not timepoint ($p = 0.47$) on daytime SpO_2 at high altitude and a Bonferroni *post hoc* revealed a mean increase of 3.55% in the probiotic group. Error bars represent standard error (SE). (B) A two-way ANOVA also revealed a significant effect of treatment group ($p = 0.011$) but not timepoint ($p = 0.66$) on mean nocturnal SpO_2 at high altitude and a Bonferroni *post hoc* revealed a mean increase of 5.07% in the probiotic group. Error bars represent SE.

Acute Mountain Sickness survey score

At sea level, all individuals had an AMS score of two or less, with most individual scores at zero (Table 1). The placebo group experienced a significant increase in AMS score from sea level to the first day at high altitude, while the probiotic group did not (p = 0.0022 and p = 0.10, paired student's t-test, respectively). Compared to the placebo group (4.9 ± 2.4), the probiotic group (2.3 ± 1.8) had a lower AMS score during the first two days at high altitude (p = 0.0026, two-way ANOVA with Bonferroni *post hoc*, ES = 1.2, 95% CI [-4.1, -0.9], Fig. 2) with a mean decrease of 2.5 but no effect of timepoint during the first two days at high altitude (p = 0.58, two-way ANOVA). Mean nocturnal SpO_2 from the previous night at high altitude did not predict AMS score of the following day in a linear model that included treatment group, age, sex, and BMI for all three nights.

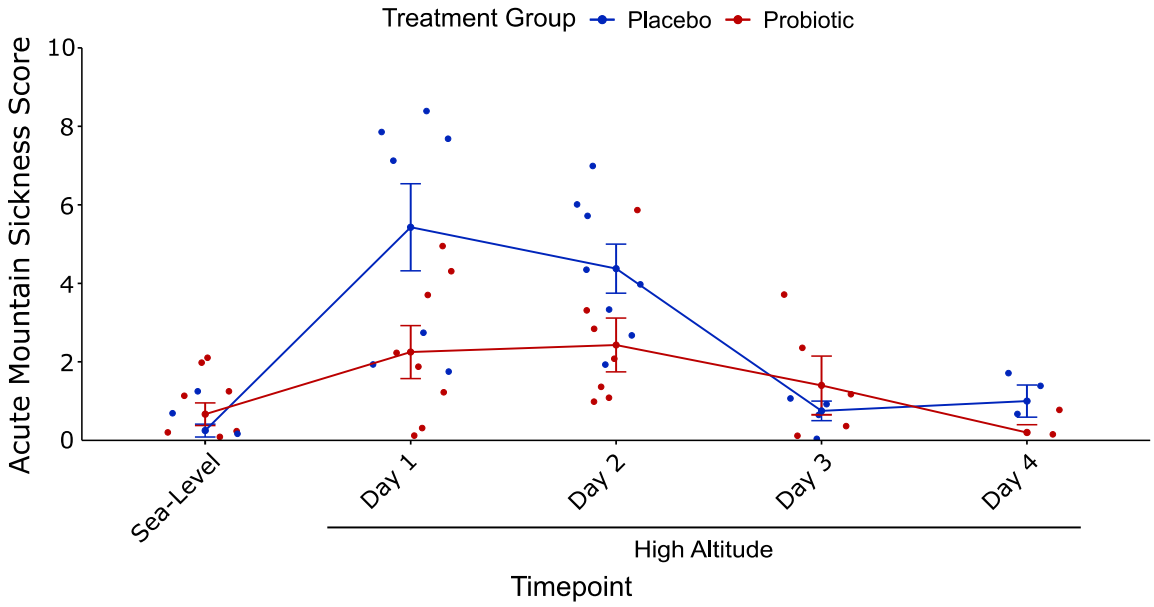


Figure 3.2: The probiotic group had decreased Acute Mountain Sickness scores compared to the placebo group during the first two days at high altitude. All individuals had an AMS score of two or less at sea level. The placebo group experienced a significant increase in AMS scores from sea level to the first day at high altitude (p = 0.0022, paired student's t-test) while the probiotic group did not (p = 0.10, paired student's t-test). The probiotic group had a lower AMS score during the first two days at high altitude compared to the placebo group (p = 0.0026, two-way ANOVA with Bonferroni *post hoc*) with a mean decrease of 2.5. Error bars represent SE.

Ventilatory and heart rate measurements during hypoxia, hypercapnia, and simultaneous hypoxia-hypercapnia

Ventilatory responses to hypoxia were measured at sea level and again on the second day (first full day) at high altitude in all participants. Both acute hypoxia and treatment group had a significant effect on $\dot{V}_I$, and the $\dot{V}_I$ measured during acute hypoxia at high altitude was significantly higher in the probiotic (0.276 ± 0.047 L/min per Kg) compared to the placebo group (0.207 ± 0.070 L/min per Kg; p = 0.012, two-way ANOVA with Bonferroni *post hoc*, ES = 1.2, 95% CI [0.014, 0.124], Fig. 3A). We did not find differences in $\dot{V}_I$ during exposure to normoxic gas (p = 0.40, two-way ANOVA with Bonferroni *post hoc*, Fig. 3A). We further analyzed the slopes of these responses to determine the HVR ($\Delta\dot{V}_I$ / ΔSpO_2), which exhibited a trend but was not significantly different between probiotic and placebo groups (p = 0.062, unpaired t-test, Fig. 3B,).

We also quantified the effects of increased levels of CO_2, and found that both acute hypercapnia and treatment group had a significant effect on $\dot{V}_I$. However, the post-hoc analysis did not show significant differences, and only a trend of higher $\dot{V}_I$ was noted in the probiotic group during acute hypercapnia (0.241 ± 0.034 L/min per Kg and 0.200 ± 0.043 L/min per Kg for probiotic and placebo groups, respectively, p = 0.054, unpaired t-test, Fig. 3C) with no significant differences in HCVR (p = 0.22, unpaired t-test, Fig. 3D).

Exposure to hypoxic-hypercapnia produced an increased level of $\dot{V}_I$ compared to the values observed for acute hypoxia alone (values of $\dot{V}_I$ observed in acute hypoxia alone are included in faded circles for reference in Fig. 3E). Both treatment group and simultaneous hypoxia-hypercapnia had significant effects on $\dot{V}_I$. Our analysis revealed a significant increase of $\dot{V}_I$ during

hypoxia-hypercapnia for participants who consumed probiotic (0.392 ± 0.064 L/min per Kg) compared to placebo (0.282 ± 0.096 L/min per Kg) at high altitude (p = 0.002, two-way ANOVA with Bonferroni *post hoc*, ES = 1.4, 95% CI [0.04, 0.18], Fig. 3E). Participants who received the probiotic had a greater hypercapnic HVR (0.020 ± 0.008 $\Delta\dot{V}_I$ / ΔSpO_2 per Kg) compared to placebo (0.012 ± 0.009 $\Delta\dot{V}_I$ / ΔSpO_2 per Kg, p = 0.036, unpaired t-test, Fig. 3F).

Similar analyses of the HR response during hypoxia, hypercapnia, and combined hypoxia-hypercapnia showed that the HR response did not differ by treatment group (Supplementary Fig. 2A-C).

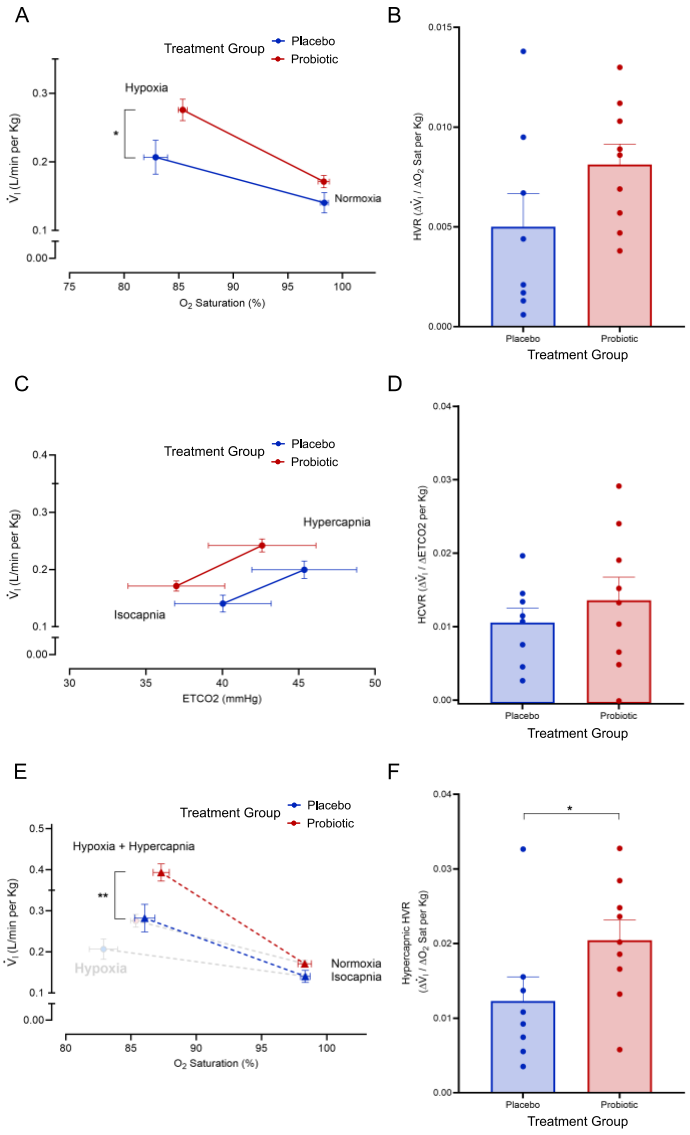


Figure 3.3: The probiotic group exhibited higher $\dot{V}_I$ during acute hypoxia exposure and higher hypercapnic HVRs at high altitude compared to the placebo group. The probiotic group (A) exhibited a higher $\dot{V}_I$ during acute hypoxia at high altitude compared to the placebo group (p = 0.012, unpaired t-test) but (B) did not have a significantly higher HVR at high altitude (p = 0.062, unpaired t-test). There were no significant differences between the treatment groups for (C) $\dot{V}_I$ during acute hypercapnia (p = 0.054, unpaired t-test) and (D) HCVR at high altitude (p = 0.22, unpaired t-test). (E) The probiotic group exhibited a higher $\dot{V}_I$ during combined hypercapnia-hypoxia (p = 0.002, unpaired t-test) compared to the placebo group and both groups exhibited a higher $\dot{V}_I$ in combined hypercapnia-hypoxia compared to just hypoxia (depicted in faded). (F) The probiotic group also exhibited a higher hypercapnic HVR at high altitude compared to the placebo group (p = 0.036, unpaired t-test). One asterisk (*) represents $p < 0.05$ and two asterisks (**) represents $p < 0.01$. All error bars represent SE.

Sleep measurements

At sea level, individuals who were in the placebo and probiotic group did not exhibit differences in the AHI ($p = 0.72$, student's t-test, Table 1) and the ODI ($p = 0.83$, student's t-test, Table 1). The cohort of 17 individuals experienced an increase in AHI upon ascent to high altitude after ingesting the probiotic or placebo ($p = 0.0078$ for placebo group, $p = 0.0012$ for probiotic group, paired student's t-tests, Fig. 4A) as well as ODI ($p = 0.00096$ for placebo group, $p = 0.00089$ for probiotic group, paired student's t-tests, Fig. 4B). However, there was no difference in AHI ($p = 0.83$, two-way ANOVA, Fig. 4A) and ODI ($p = 0.73$, two-way ANOVA, Fig. 4B) between the two groups. Neither AHI nor ODI from the first night at high altitude predicted AMS score of the following day in a linear model that included treatment group, age, sex, and BMI.

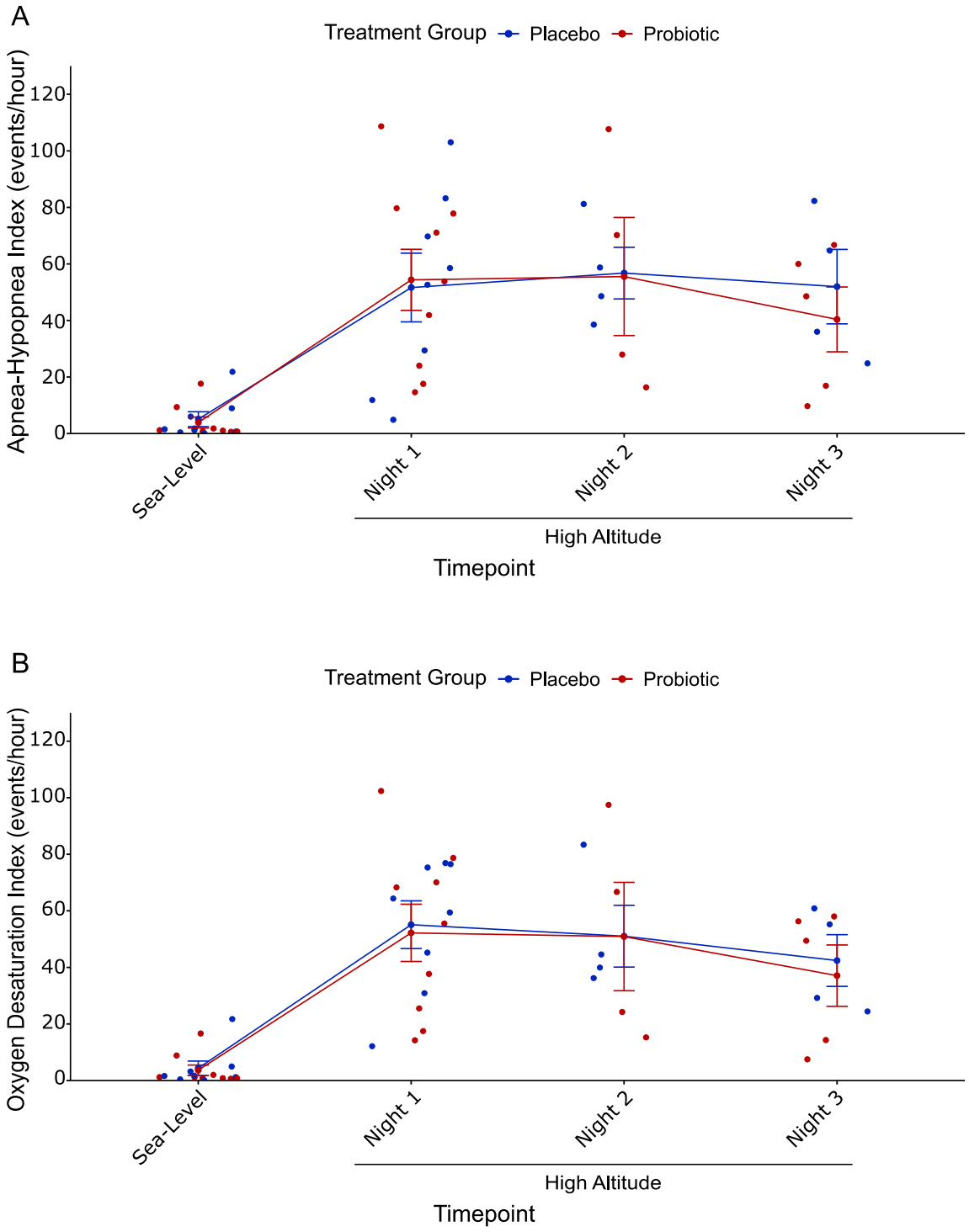


Figure 3.4: Treatment groups did not exhibit any differences in AHI or ODI at high altitude. Both treatment groups experienced an increase in (A) AHI (p = 0.0078 for placebo group, p = 0.0012 for probiotic group, paired student's t-tests) and (B) ODI (p = 0.00096 for placebo group, p = 0.00089 for probiotic group, paired student's t-tests. There was no difference in (A) AHI or (B) ODI between the two groups at high altitude. Error bars represent SE.

Basic cardiovascular measurements

Prior to administration of the probiotic or placebo, resting HR increased in all individuals from sea level to high altitude (p = 0.0084 for placebo group, p = 0.0064 for probiotic group, student's t-test, Fig. 5A), and did not differ between placebo and probiotic group at both sea level (Table 1) and at high altitude (p = 0.91, two-way ANOVA, Fig. 5A). Systolic blood pressure did not differ between the two groups at sea level (p = 0.19, student's t-test, Table 1) and did not change with exposure to high altitude for either group (p = 0.36 for placebo group, p = 0.69 for probiotic group, paired student's t-tests, Fig. 5B). There was no difference in systolic blood pressure between the two groups (p = 0.22, two-way ANOVA, Fig. 5B). Diastolic blood pressure was higher in the placebo group (83.5 ± 10.9 mmHg) compared to the probiotic group (70.1 ± 8.05 mmHg) at sea level (p = 0.014, student's t-test, ES = 1.40, 95% CI [3.24, 23.6], Fig. 5C) and decreased with exposure to high altitude in the placebo group (78.4 ± 10.3 mmHg, p = 0.045, student's paired t-test, ES = 0.48, 95% CI [0.149, 10.1], Fig. 5C), but not the probiotic group (p = 0.57, student's paired t-test, Fig. 5C). There was no difference in diastolic blood pressure between the two groups at high altitude (p = 0.92, two-way ANOVA, Fig. 5C).

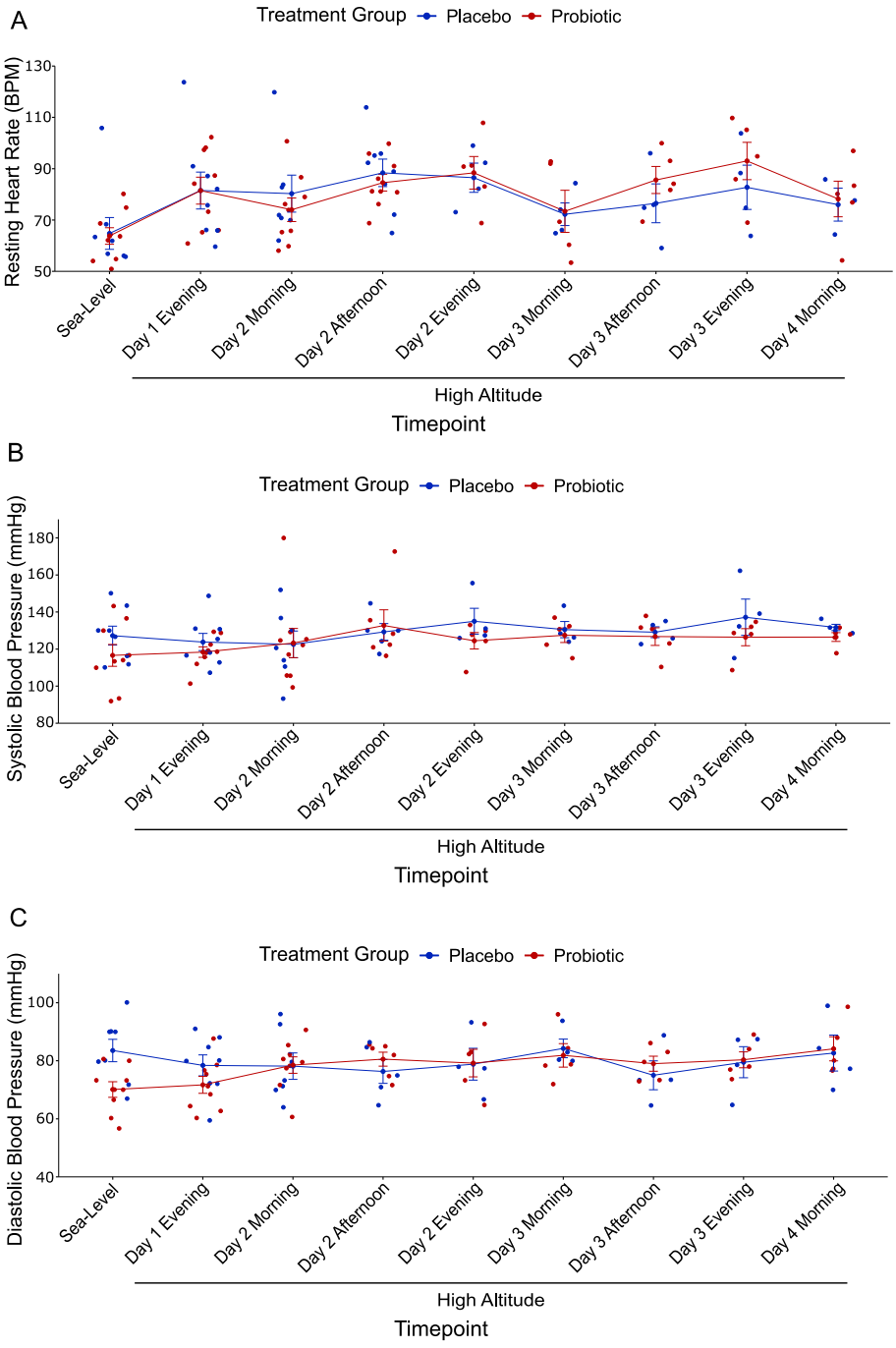


Figure 3.5: Treatment groups did not exhibit any differences in resting heart rate, systolic blood pressure, or diastolic blood pressure at high altitude. (A) Both groups experienced an increase in resting HR from sea level to high altitude ($p = 0.0084$ for placebo group, $p = 0.0064$ for probiotic group, student's t-test) but did not differ in resting HR at high altitude. Error bars represent SE. (B) There was no difference in systolic blood pressure from sea level to high altitude in either group and no difference in systolic blood pressure between the two groups at high altitude. (C) Diastolic blood pressure decreased with exposure to high altitude in the placebo group ($p = 0.045$, student's paired t-test) but did not change in the probiotic group. There was no difference in diastolic blood pressure between treatment groups at high altitude. Error bars represent SE.

Heart rate variability

We completed analysis of HRV to obtain time-domain SDRR and RMSDD parameters, as well as frequency-domain LF, HF, and LF/HF values. In the entire group of participants, SDRR was significantly lower during the first morning at high altitude (62.0 ± 27.9 ms) compared to sea level (84.6 ± 36.1 ms, $p = 0.028$, student's t-test, Fig. 6A), and RMSDD did not differ between sea level and the first morning at high altitude ($p = 0.074$, student's t-test, Fig. 6B). Although there were no significant effects of altitude in the full cohort for LF ($p = 0.081$, student's t-test, Fig. 6C) or HF ($p = 0.155$, student's t-test, Fig. 6D), there was a significant increase in LF/HF at high altitude (2.58 ± 1.72) compared to the values obtained at sea level (1.62 ± 0.92, $p = 0.03$, student's t-test, Fig. 6E). There were no significant differences in LF/HF between treatment groups at high altitude ($p = 0.12$, two-way ANOVA, Fig. 6E), time-domain parameters of SDRR and RMSDD (p =0.21 and 0.10 respectively, two-way ANOVA, Fig. 6A and 6B), and frequency-domain parameters of LF ($p = 0.20$, two-way ANOVA, Fig. 6C) and HF ($p = 0.196$, two-way ANOVA, Fig. 6D).

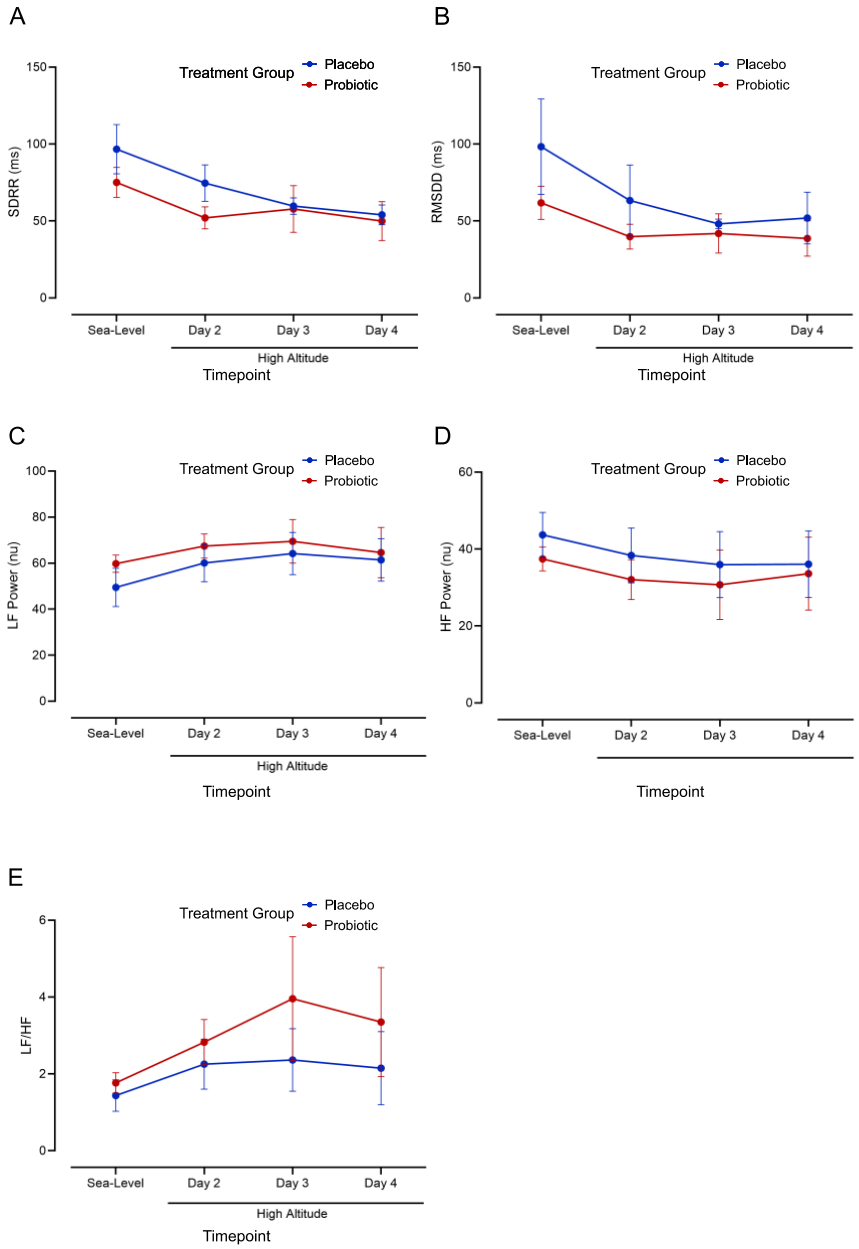


Figure 3.6: Treatment groups did not exhibit differences in measures of heart rate variability during exposure to high altitude. (A) Both groups experienced a decrease in SDRR during the second day at high altitude compared to sea level (p = 0.028, student's t-test) but no differences between treatment groups (p = 0.21, two-way ANOVA). (B) Neither group exhibited a change in RMSDD with exposure to high altitude (p = 0.074, student's t-test) nor was there a difference between treatment groups in RMSDD (p = 0.10, two-way ANOVA). (C and D) There were no differences in LF or HF between sea level and the second day at high altitude in both groups (p = 0.081 and p = 0.155, respectively, student's t-test) and no differences between groups at high altitude for both LF (p = 0.20, two-way ANOVA) and HF (p = 0.20, two-way ANOVA). (E) Both groups experienced an increase in LF/HR ratio during the second day at high altitude compared to sea level (p = 0.03, student's t-test) but no differences between treatment groups (p = 0.12, two-way ANOVA). All error bars represent SE.

Fasting glucose

Fasting glucose did not significantly change from sea level (Table 1) to high altitude in either group but did, on average, increase in the placebo group by a mean of 9.25 mg/dL (p = 0.20, student's t-test, Fig. 7) and decrease in the probiotic group by a mean of 5.11 mg/dL (p = 0.16, student's t-test, Fig. 7). Fasting glucose did not differ between the placebo and probiotic group at high altitude (p = 0.091, two-way ANOVA, Fig. 7), but the probiotic group did trend towards lower fasting glucose levels on the second day at high altitude (p = 0.063, student's t-test, Fig. 7).

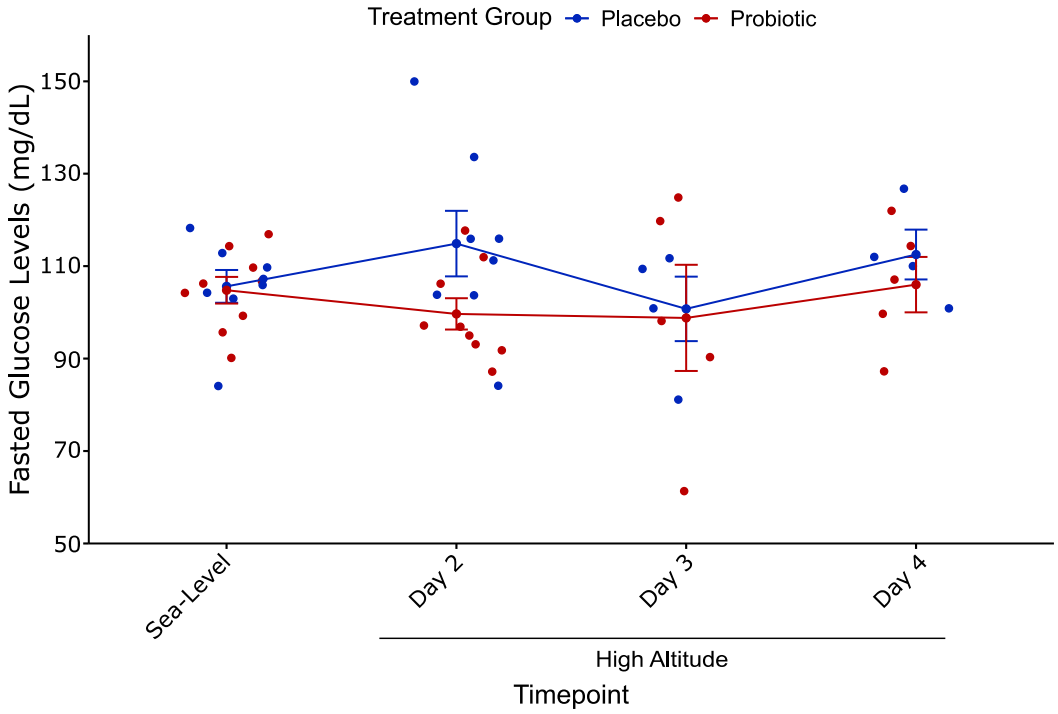


Figure 3.7: Treatment groups did not exhibit differences in fasted glucose levels at high altitude. Both treatment groups did not experience a significant change in fasted glucose levels from sea level to the first morning (Day 2) at high altitude and did not differ in fasted glucose levels at high altitude. However, the probiotic group did trend towards lower fasted glucose levels on the first morning at high altitude (p = 0.063, student's t-test). Error bars represent SE.

Discussion

This study provides the first evidence that ingestion of the probiotic SLAB51 has the capability to improve acclimatization to high altitude in humans. We explored the impact of SLAB51 on high-altitude acclimatization in sea-level residents in a randomized, placebo-controlled, double-blinded interventional study. The probiotic had never been tested in this context before, though previous studies provide evidence that SLAB51 may improve oxygenation in COVID-19 patients (Ceccarelli et al., 2021) and preterm infants (Baldassarre et al., 2023). Our findings show that SLAB51 ingestion increases daytime and nighttime O_2 saturation, decreases symptoms of AMS, and increases ventilation. We also measured AHI, ODI, resting HR, blood pressure, HRV, and fasting glucose, all of which did not differ significantly between the two treatment groups.

The probiotic group had higher daytime SpO_2 than the placebo group at high altitude with a mean increase of 3.6% (Fig. 1A). When predicting daytime SpO_2 in a mixed-effects model considering other factors such as ventilation, age, sex, and BMI, the effect size of the probiotic was 2.5%, with the model indicating that HVR at high altitude is also a significant contributor to daytime SpO_2 (Supplementary Table 1). While the HVR is known to lessen the reduction in SpO_2 one would otherwise experience due to hypobaric hypoxia (Oeung et al., 2023), the mixed-effects model indicates that this specific probiotic also has an independent contributing effect on SpO_2. While the direct mechanism might involve O_2 preservation as suggested (Ceccarelli et al., 2021), it is plausible alterations in small molecule and/or gaseous signals result in altered physiological effects (Ju et al., 2020; Khoo, Koh et al., 1996). In any case, the mechanism of action quickly affects O_2 saturation as SpO_2 measurements were taken approximately two to three hours after the first ingestion at high altitude. These values are greater than the increase in SpO_2 seen with

acetazolamide when ascending to high altitude in studies conducted at 4,937 m and 3,658 m, which report an 1.8% (Basnyat et al., 2003) and 2.5% (Ke et al., 2013) improvement in SpO_2, respectively, though it is important to note that the ascent profiles varied in these studies and compared to ours.

The probiotic group also had a higher nighttime SpO_2 than the placebo group at high altitude with an even higher mean increase than the daytime SpO_2 of 5.1% (Fig. 1B). This value is similar to the increase in nocturnal SpO_2 seen with acetazolamide in a meta-analysis that reported a mean increase of 4.75% in nocturnal SpO_2 (Liu, H.-M. et al., 2017). At high altitude, humans experience a reduction in SpO_2 during sleep due to decreased ventilatory drive (Ju et al., 2020; Khoo, Koh et al., 1996) and many experience sleep-disordered breathing (SBD) that further depresses nighttime SpO_2 (Ainslie et al., 2013; Khoo, Anholm et al., 1996). These depressions in SpO_2 during sleep can result in a sequelae of negative physiological health impacts, such as inflammation, increased sympathetic activation, oxidative stress, and increased risk of cardiovascular disease (Benjafield et al., 2019; Dempsey et al., 2010; Mesarwi et al., 2015; Sajkov et al., 1999). A previous study indicated that SLAB51 may reduce inflammation and oxidative stress caused by chronic sleep restriction in mice (Zheng, Y. et al., 2023), and this study indicates that the same probiotic may also attenuate the reduction in nighttime SpO_2 experienced by humans during sleep at high altitude, though with minimal effects on SDB itself (discussed further below).

Compared to the placebo group, the probiotic group had a lower AMS score during the first two days at high altitude (Fig. 2) with a mean decrease in score of 2.5. The Lake Louise Acute AMS survey asks participants to assess severity of their AMS symptoms on a number scale (Roach, R. C. et al., 2018) and has been utilized by a wide range of studies to assess AMS symptom severity (Boos et al., 2016; Julian et al., 2014; Roach, R. C. et al., 2018; Seiler et al., 2023). Many

humans experience AMS symptoms when they ascend to high altitudes, and the severity of the symptoms are usually highest on the first and second days at elevation (Imray et al., 2010; Singh, I. et al., 1969). This study indicates that consumption of the probiotic SLAB51 may reduce the severity of AMS symptoms as evidenced by lower AMS scores in the probiotic group when compared to the placebo group. How exactly the probiotic may alleviate AMS symptoms is unknown, though a higher SpO_2 is known to be associated with a lower AMS score (Boos et al., 2016). Within our cohort, mean nocturnal SpO_2, AHI, or ODI did not predict AMS score the following day in a mixed effects model that included treatment group, age, sex, and BMI. Previous studies had indicated that sleep disturbance at high altitude did not correlate with AMS severity the following day (Hall, D. P. et al., 2014; Macinnis et al., 2013), which prompted a change in the Lake Louise scoring of AMS severity and removed sleep as part of the questionnaire in 2018 (Roach, R. C. et al., 2018). Although limited to measurements of mean nocturnal SpO_2, AHI, and ODI, which may not capture pivotal patterns of hypoxemia during sleep (Malhotra, A. et al., 2021; Trzepizur et al., 2021), our results support this notion based on the measurements obtained.

The probiotic group had a higher $\dot{V}_I$ during the hypoxic stage of the ventilation protocol than the placebo group (Fig. 3A), but the overall HVRs were not significantly different between the two groups (Fig. 3B). Ventilation increases with exposure to hypoxia (Pamenter and Powell, F. L., 2016; Teppema and Dahan, 2010), and this increase in ventilation is a strong indicator of acclimatization to the hypoxia experienced at high altitude (Mallet et al., 2023). Because ventilation is a key contributor to SpO_2 (Pamenter and Powell, F. L., 2016; Sato et al., 1992), we quantified ventilation in room air, normoxia, and hypoxia at high altitude. Our mixed-effects model predicting daytime SpO_2 indicates that HVR at high altitude did indeed significantly predict daytime SpO_2 (Supplementary Table 1), though this was found to be independent of treatment

group. A higher $\dot{V}_I$ during the hypoxic stage in the probiotic group at high altitude compared to the placebo group indicates SLAB51 probiotic consumption may increase ventilation, but this difference did not extend to the HVRs of the respective groups, though there was a trend toward higher HVRs in the probiotic group (Fig. 3B). The HVR is highly variable among individuals (Oeung et al., 2023), and a larger sample size may be able to detect a significant difference between treatment groups. It is also feasible that there may be interactions among the immune system, ventilation, and probiotic consumption. Basaran et al. (2016) found that ibuprofen decreased the HVR in humans after 48 hours at high altitude. This result was previously investigated in rats, where administration of ibuprofen prevented the hypoxia-dependent increase of ventilation and levels of inflammatory makers in the nucleus tractus solitarii (involved in control of breathing) (Popa et al., 2011). Although all the bacteria in SLAB51 are gram-negative, consumption of live bacteria may elicit an immune response, though more studies must be conducted to determine if this occurs. These factors may contribute to the fast-acting effect that probiotic administration had on O_2 saturation.

An analysis of HCVR at sea level and high altitude showed no significant differences in HCVR between treatment groups at high altitude (Fig. 3D), though there was a trend toward higher $\dot{V}_I$ during hypercapnia in the probiotic group (Fig. 3C). HCVR is believed to play a role in SDB susceptibility at high altitude–increased sensitivity to CO_2 during sleep may drive increased respiration and exhalation of CO_2, leading to central sleep apneas, though this potential connection requires further study (Ainslie et al., 2013; Masuyama et al., 1989). A previous study provides evidence that manipulation of the gut microbiota affects the HCVR in rats (Schultz et al., 2014; Vogel and Harris, C. W., 1967; Yan, Y. et al., 2023), warranting further investigations into a potential connection between the gut microbiota and ventilatory responses via the gut-lung axis.

An analysis of combined hypoxia and hypercapnia on ventilation, also known as the hypercapnic HVR, revealed a significantly higher hypercapnic HVR in the probiotic group compared to the placebo group at high altitude (Fig. 3F). The combination of hypoxia and hypercapnia produces a synergistic effect (Teppema and Dahan, 2010); therefore, while hypoxic and hypercapnic signals alone were close to significant, discernible differences in ventilatory responses between treatment groups was detected during the hypoxic hypercapnic stage of the protocol. An analysis of the HR response during the HVR, HCVR, and hypercapnic HVR revealed no differences between treatment groups (Supplementary Figure 2. In combination, these results suggest the SLAB51 probiotic may augment ventilatory, but not cardiac, responses to O_2 and CO_2, which warrants further investigation.

Although both the placebo and probiotic groups experienced an increase in AHI and ODI from sea level to high altitude (Fig. 4A and Fig. 4B, respectively), there was no difference between the two groups at high altitude for both measurements (Fig. 4A and Fig. 4B). These results suggest that the differences observed for nocturnal SpO_2 between the two groups were not primarily driven by these metrics of SDB, and that SLAB51 may not alleviate these patterns of intermittent hypoxia experienced with SDB at high altitude. When humans ascend to high altitude, they often experience intermittent hypoxia during sleep in addition to sustained hypoxia (Vogel and Harris, C. W., 1967), and this combination may lead to poor cardio-metabolic outcomes (Zhen et al., 2021). Therefore, while SLAB51 may not ameliorate SDB and intermittent hypoxia exposure, it may mitigate the extent of hypoxia experienced at high altitude, thereby still having a positive impact on cardio-metabolic health.

Resting HR of both groups increased with exposure to high altitude but did not differ between the two groups at high altitude (Fig. 5A). This increase in resting HR from sea level to

high altitude has been previously observed (Schultz et al., 2014; Vogel and Harris, C. W., 1967; Yan, Y. et al., 2023). Systolic blood pressure did not change with high-altitude exposure, which contrasts with other studies that exposed sea-level residents to high altitude (Schultz et al., 2014; Yan, Y. et al., 2023), though an earlier study noted no change in blood pressure with high-altitude exposure in sea-level residents (Vogel and Harris, C. W., 1967), and there was no difference between the placebo and probiotic groups (Fig. 5B). Diastolic blood pressure was higher in the placebo group at sea level and decreased with high-altitude exposure in the placebo group but didn't change in the probiotic group, resulting in no overall difference in diastolic blood pressure at high altitude between the two groups (Fig. 5C), and this initial difference in diastolic blood pressure at sea level may be due to chance.

Exposure to high altitude induced changes in HRV, indicated by lower measures of SDRR and higher levels of LF/HF compared to sea-level values (Fig. 6A and 6E). However, we did not observe any differences in HRV between treatment groups (Fig. 6). HRV is a measurement that approximates sympathetic autonomic nervous system activation (Hou et al., 2023). Previous studies indicate that hypoxia increases sympathetic activation as showcased by measures of HRV at high altitude (Insalaco et al., 2016; Perini et al., 1996) and in hypobaric chambers (Basualto-Alarcón et al., 2012; Zhang, D. et al., 2014) as well as with direct measurements of muscle sympathetic nerve activity in lowlanders travelling to high altitude and populations with a history of residence at high altitude (Deng, S. et al., 2022; Massin et al., 2000; Sammito, S. et al., 2016). Several previous studies have studied the effects of probiotic usage on HRV, but none used the particular probiotic from this study. Kikuchi-Hayakawa et al. (2023) found that probiotic containing *Lacticaseibacillus paracasei* strain Shirota decreased values of LF/HF measured during the afternoon in office workers and Quero-Calero et al. (2022) found no effects of probiotic usage

on HRV in athletes and people with sedentary habits. Another study found a positive association between *Lachnospiraceae incertae sedis* counts and higher SDRR in a general Japanese cohort (Tsubokawa et al., 2022). While we controlled for known circadian rhythm effects on HRV (Deng, S. et al., 2022; Massin et al., 2000; Sammito, S. et al., 2016) by only performing our HRV measurements in fasted conditions in the morning, it is unclear how much existing bacterial compositions may have contributed to the variability seen in HRV measurements within our cohort.

Fasting glucose levels did not increase in either group with high-altitude exposure (Fig. 7) in contrast with previous studies that exposed sea-level residents to high altitude (Larsen et al., 1997; Sawhney et al., 1991). Interestingly, the placebo group exhibited a trend toward an increase in fasting glucose levels with high-altitude exposure while the probiotic exhibited a decreased trend. This observation, in conjunction with the observed trend towards a lower fasting glucose level in the probiotic group on the second day at high altitude (Fig. 7), suggests that the probiotic may improve glucose intolerance at high altitude. This suggestion is further supported by a previous study that showed improvement in glucose uptake in Alzheimer's disease mouse models with SLAB51 treatment (Bonfili et al., 2020).

While this study is the first to investigate SLAB51 in humans acclimatizing to high altitude, it comes with limitations. Although we detect a significant difference between the two treatment groups for our main outcome SpO_2 during both daytime and nighttime, our sample size of 17 individuals is limited and could be expanded in future studies. Additional investigation into how SLAB51 improves O_2 saturation is also warranted. Although some evidence suggests modulation of the ventilatory responses to hypoxia (Fig. 3), the mixed effects model predicting daytime SpO_2 indicates that the probiotic also has a positive effect on SpO_2 that is separate from the HVR. A

previous study suggests SLAB51 ingestion may stabilize HIF-1α in human intestinal epithelial cells exposed to lipopolysaccharide (Lombardi et al., 2023). Another potential mediating mechanism is through short-chain fatty acids (SCFAs). SCFAs, while typically associated with distal microbiota and bacterial colonization not involved in the fast-acting effects described, are known to facilitate communication between microbial cells and host cells, including neurons (Oleskin and Shenderov, 2016). Butyrate is a SCFA utilized by human intestinal epithelial cells as an energy source and also directly inhibits prolyl hydroxylases (PHDs), in turn helping to stabilize HIF1A (Wang, R. X. et al., 2021). It may be feasible that SLAB51 ingestion may interact with the proximal intestine and increase SCFA secretion, such as butyrate, thereby having a stabilizing effect on HIG1A. Additionally, gaseous transmitters linked to the microbiota, such as carbon monoxide (CO) and hydrogen sulfide (H_2S) with further implications for nitric oxide (NO) (Oleskin and Shenderov, 2016), are associated with the control of breathing metrics in animal models (Peng, Y.-J. et al., 2017), which we hypothesize influence patterns of breathing during the daytime and also during sleep in humans. Further studies investigating the mechanism of SLAB51 in healthy humans is warranted.

Current methods to improve acclimatization to high altitude include slow and steady ascent to altitude (Imray et al., 2010) and/or use of acetazolamide (Basnyat and Murdoch, 2003; Leaf and Goldfarb, 2007). This study provides evidence for a novel, non-invasive intervention to improve acclimatization to high altitude within a relatively short period of time by ingestion of a specific probiotic. Furthermore, this study suggests that targeted bacteriotherapy may be a previously underutilized and overlooked method of improving O_2 saturation. This application is relevant in the context provided in this study (healthy sea-level residents ascending to higher elevations, such as for recreational or employment reasons), but may also be expanded to other contexts, such as

individuals with diseases related to O_2 homeostasis, high-altitude residents, and military personnel operating in hypoxic environments. Individuals with diseases such as chronic obstructive pulmonary disease (Corlateanu et al., 2016), sickle cell disease (Obadina et al., 2023), and sleep apnea (Mesarwi et al., 2015), all struggle with O_2 homeostasis – improvement of O_2 saturation by bacteriotherapy may be a method to increase baseline SpO_2 during both daytime and nighttime. Millions of individuals already reside at high altitude (Tremblay and Ainslie, 2021) and, in a warming world, more may seek out higher altitudes as relief from climate change. As non-adapted individuals, they may benefit from interventions that improve O_2 saturation. Even populations with high-altitude ancestry of hundreds of generations who have adapted to their local environments suffer from maladies related to high altitude, such as Chronic Mountain Sickness (Villafuerte et al., 2022). Acetazolamide has been previously tested for its efficacy in combatting Chronic Mountain Sickness (Champigneulle et al., 2023; Sharma, S. et al., 2017), and bacteriotherapy may be a new tool to add to the toolkit. Lastly, military operations can occur in high-altitude regions where altitude sickness can be a serious detriment (Peoples et al., 2005), and military aviators constantly face the threat of hypoxia (Shaw et al., 2021). Therefore, targeted bacteriotherapy provides a novel avenue to help combat the threat of hypoxia in various environments and potential pathological conditions.

Author Contributions

JJY: experimental design; acquisition of data; processing of data; data and statistical analysis and interpretation of data; drafting of the original manuscript. EAM: experimental design; acquisition of data; processing of data; data and statistical analysis and interpretation of data; drafting of the original manuscript. HC: acquisition of data; processing of data. KK: acquisition of

data; processing of data. TO: acquisition of data; processing of data. SF: acquisition of data. EG: acquisition of sleep data; processing of sleep data. AS: acquisition of sleep data; processing of sleep data. PD: processing of sleep data. EVY: acquisition of data. LAB: acquisition of data. AL: acquisition of data. AS-A: acquisition of data. JEO: medical oversight of study; interpretation of data. ECH: acquisition of data. AM: oversight of sleep data collection. TSS: experimental design; acquisition of data; interpretation of data; drafting and revision of the original manuscript.

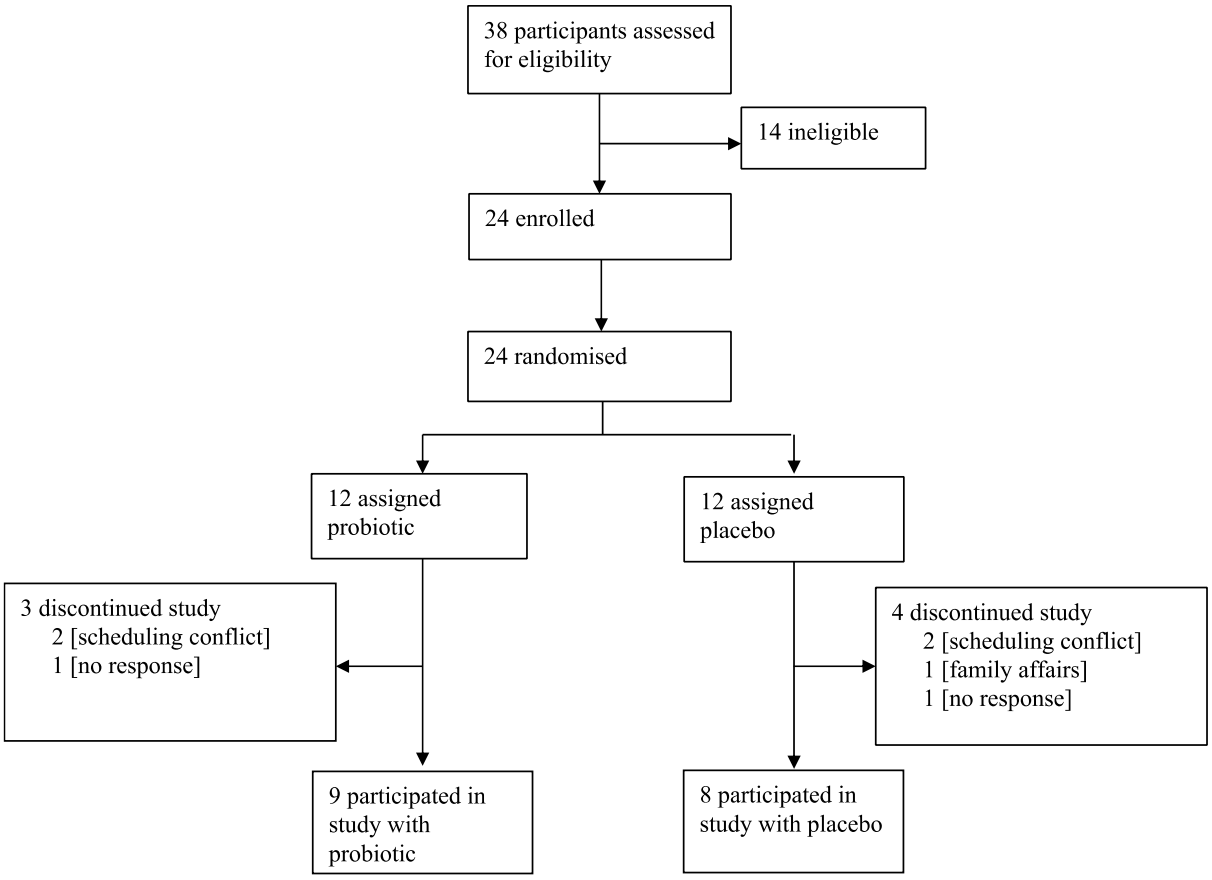
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Chapter 3, in full, is currently being prepared for submission for publication of the material. Yu, James J.; Moya, Esteban A.; Cheng, Hunter; Kaya, Kiana; Ochoa, Tim; Fassardi, Santiago; Gruenberg, Eli; Spenceley, Alex; DeYoung, Pamela; Young, Elizabeth V.; Barnes, Laura A.; Lugo, Alina; Sanchez-Azofra, Ana; Orr, Jeremy E.; Heinrich, Erica C.; Malhotra, Atul; Simonson, Tatum S. The dissertation author was co-first author of this paper. The authors thank the staff of the White Mountain Research Center for their support at Barcroft Station. We thank Frank Powell for providing expertise in experimental design and results interpretation and Janelle Fine for overseeing the blinding of the probiotic and placebo, and also fixing all hardware issues encountered throughout the study. We thank Prof Claudio de Simone for instruction on storage of SLAB51.

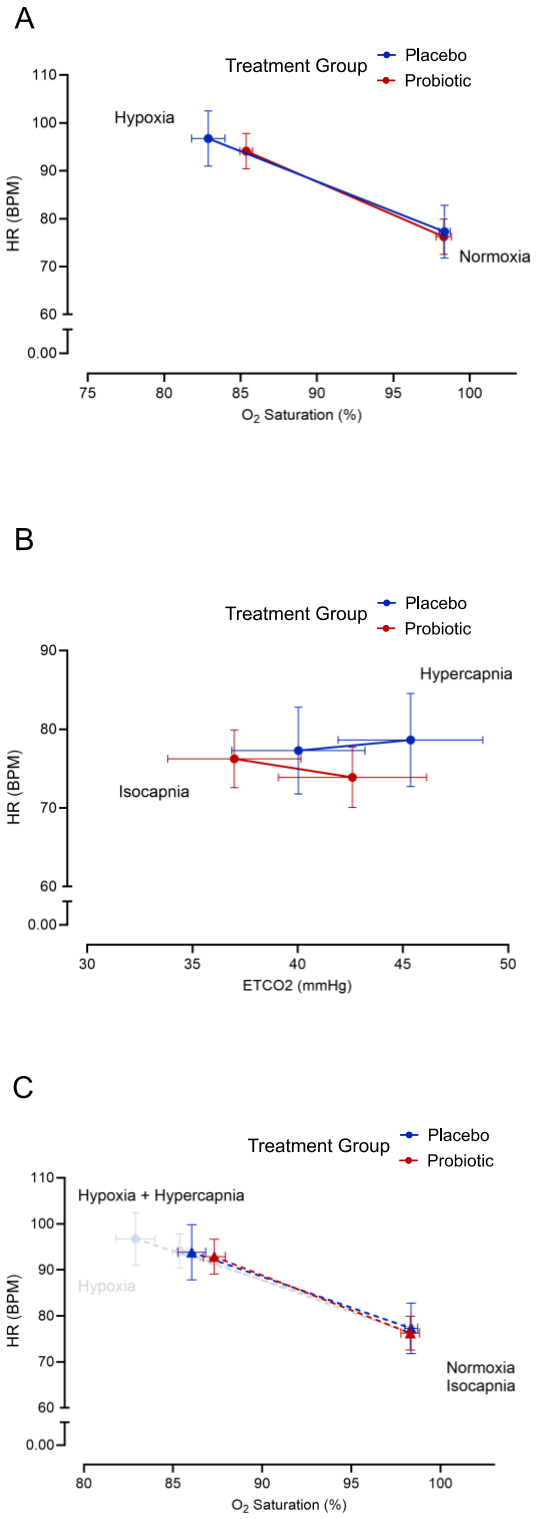
Supplemental Table 3.1: Statistical summary of mixed effects model predicting daytime SpO_2 at high altitude.

effect	group	term	estimate	std.error	statistic
fixed		(Intercept)	81.94435406	3.87285291	21.1586538
fixed		Treatment_Probiotic	2.51026390	1.08636442	2.3107015
fixed		Ventilation_HVR	4.88064473	2.00383076	2.4356572
fixed		Age	-0.08142088	0.04632795	-1.7574893
fixed		Sex_M	1.14314393	1.40353518	0.8144747
fixed		BMI	0.15270351	0.12315996	1.2398795
ran_pars	ID	sd__(Intercept)	1.64274963		
ran_pars	Timepoint	sd__(Intercept)	0.67805624		
ran_pars	Residual	sd__Observation	2.57816016		

HVR, hypoxic ventilatory response; M, male; BMI, body mass index; SpO_2, oxygen saturation measured by pulse oximetry.



Supplemental Figure 3.1: Trial profile overview. 24 participants were enrolled in the study from 38 individuals that were assessed for eligibility. These 24 participants were randomly split into the probiotic group and the placebo group. 17 of these participants completed the study.



Supplemental Figure 3.2: Heart rate responses did not differ between treatment groups during ventilatory measurements in various hypoxic and hypercapnic stages. Heart rate responses did not differ during (A) hypoxia, (B) hypercapnia, and (C) combined hypercapnia-hypoxia between the treatment groups (two-way ANOVA) . All error bars represent SE.

CONCLUSION

The human response to high altitude is complex and variable between and within populations. Oxygen homeostasis is critical for human health and disease, therefore understanding the human response to hypoxia is paramount. Populations that have resided at high altitude for hundreds of generations have augmented responses to hypoxia, allowing a unique opportunity to investigate the human body's relationship with oxygen through various lenses. This dissertation sheds light on how two different populations, Andeans and Tibetans, have adapted to high altitude, and investigates the potential of a probiotic to augment the response to high altitude in sea-level residents.

The first chapter is the result of a multi-omics analysis in Andean highlanders combining genetic, proteomic, and metabolomic data to predict physiological phenotypes related to high-altitude adaptation. It is the first multi-omics analysis ever conducted in a high-altitude population and establishes a framework for how to utilize various datasets that will increase in accessibility as -omic technologies improve. The analysis identified biological predictors of phenotypic outcomes that can be further investigated for adaptive properties.

Chapter Two summarizes a novel association between menopausal status and ventilatory responses to hypoxia in Tibetan women, along with genetic associations to ventilatory traits and positively selected genes related to control of breathing. It is the result of extensive fieldwork conducted in Mustang, Nepal, the first GWAS ever conducted in Tibetans for ventilatory traits, and the first application of a newer positive selection scan, iSAFE, in Tibetans. The study identified lower HVRs in post-menopausal women compared to pre-menopausal, new genetic associations

with ventilatory traits, and new genetic selection candidates that may play a role in control of breathing.

The third chapter is the result of a preliminary, randomized, double-blinded, placebo-controlled study to test the effects of the probiotic, SLAB51, on acclimatization to high altitude in sea-level residents. The results show that individuals that consumed the probiotic had a higher SpO_2 and lower AMS scores at high altitude than those that consumed the placebo. This is the first example of a probiotic improving high-altitude acclimatization and paves the way for future studies to explore augmentation of the gut microbiota as an avenue to improve oxygenation and high-altitude acclimatization.

While this dissertation provides an exploration of the human response to high altitude through various lenses, the work is never complete. Multi-omics analyses depend heavily on the inputted data, and future multi-omics analyses would benefit from larger data sets, more data types, such as transcriptomics, and deeper phenotyping. The HVR studies in Tibetan women must also be conducted in various other populations, such as Andeans and sea-level residents, to determine if menopausal status also associates with ventilation in these groups. Lastly, the mechanism for SLAB51's effect of improved SpO_2 remains unclear–future studies should investigate if there are any changes to blood-gas levels, such as a shift in the oxyhemoglobin disassociation curve, oxidative stress, or oxygen demand by the intestinal tract.

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