HUMAN MOLECULAR AND PHYSIOLOGICAL RESPONSES TO HYPOXIA

EDITED BY: Sandro Malacrida, Annalisa Cogo, Simona Mrakic-Sposta, Matiram Pun and Giacomo Strapazzon

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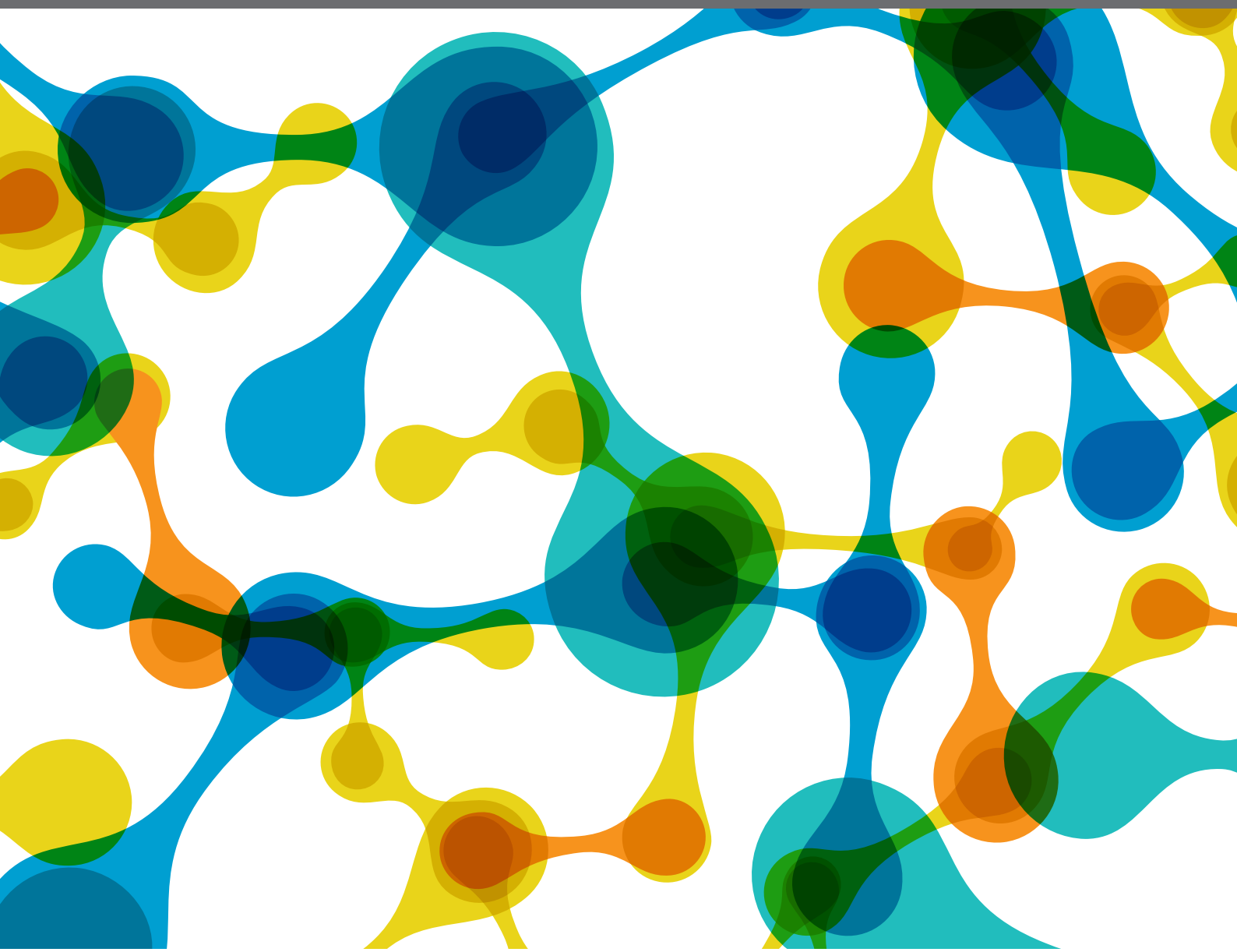

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HUMAN MOLECULAR AND PHYSIOLOGICAL RESPONSES TO HYPOXIA

Topic Editors:
Sandro Malacrida, Eurac Research, Italy
Annalisa Cogo, Istituto Pio XII, Italy
Simona Mrakic-Sposta, Italian National Research Council, Italy
Matiram Pun, University of Calgary, Canada
Giacomo Strapazzon, Eurac Research, Italy

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Table of Contents

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EDITORIAL
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Editorial: Human Molecular and Physiological Responses to Hypoxia

Sandro Malacrida[1]*, *Giacomo Strapazzon*[1], *Simona Mrakic-Sposta*[2], *Matiram Pun*[3] and *Annalisa Cogo*[4,5,6]

[1]Institute of Mountain Emergency Medicine, Eurac Research, Bolzano, Italy, [2]Institute of Clinical Physiology—National Research Council IFC—CNR, Milan, Italy, [3]Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada, [4]University of Ferrara, Ferrara, Italy, [5]Center for Exercise and Sport Science University of Ferrara, Ferrara, Italy, [6]Institute Pio XII, Misurina, Italy

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Editorial on the Research Topic

Human Molecular and Physiological Responses to Hypoxia

Towards the end of the 19th century, the French physician Denis Jourdan was the first to understand and state the critical role of the reduction of oxygen at altitude, which he defined as anoxemia. This term indicated the diminished quantity of oxygen contained in the blood of people living at high altitude, where the tension of the oxygen in the surrounding air is considerably decreased (West and Richalet, 2013). In the following 150 years, studies on hypoxia took off, ranging from purely clinical and functional aspects to cellular and biomolecular ones, from acute to chronic hypoxia and analyzing not only the altitude-hypoxia but also the hypoxia related to underlying diseases. Currently, the study of pathophysiological responses at altitude is a model to investigate the mechanisms of response to hypoxia in any condition, also in critical illnesses (Grocott et al., 2007).

In this special issue, a series of ten articles with different approaches applied to the study of molecular and physiological responses to hypoxia were collected.

A crucial question is the effect of prolonged hypoxia on circulating plasma lipid profile, more specifically its capacity to increase plasma triglyceride (TG) concentrations. In animal models, hypoxia has been shown to have detrimental effects on many aspects of triglyceride metabolism. Rene Morin et al. wrote a mini review highlighting that hypoxia tends to negatively affect TG levels by increasing the concentration of denser triglyceride-rich lipoproteins, mainly in prandial and postprandial states. These results can help to develop strategies to mitigate the effect of hypoxia on TG levels and the burden of a possible increase of cardiovascular risk. Ortiz-Prado et al. in fact, compared two populations with similar genetical, sociodemographic and economical characteristic, but living at different altitudes. They could, therefore, focus on the difference given by the altitude. They reconfirmed the well-known adaptive physiological changes related to life at altitude and they reported clinical differences in the plasma lipid profile, with higher levels of cholesterol and high density and low-density lipoproteins in Andinean population versus the Amazonian-basin one. The 10-years risk for cardiovascular disease was not different between the two groups. Pooja Kumarie et al. investigated the response to long-term high-altitude exposure on physiological indices, pro-inflammatory cytokines (IL-6, TNFα and CRP) and plasma proteome in 105 healthy male military subjects at sea level and after a short (7 days) at 3,520 m, and a long-term stay (3 months) at 4,176 m. Plasma proteomics studies revealed higher levels of apolipoproteins (APOB, APOCI, APOCIII, APOE, and APOL), carbonic anhydrases (CA1 and CA2) and proinflammatory cytokines during hypoxia exposure. These results suggest a vascular inflammation and demonstrate that long-term stay at high altitude exacerbate dyslipidemia and associated disorders.

Effects of hypoxia on different organs and populations has been investigated in different studies. Pedreros-Lobos et al. studied a group of miners at sea level and at moderate altitude. Despite the well-known decrease of VO_2 max at altitude, they found that work capacity, heart rate, and

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Richard D. Boyle, National Aeronautics and Space Administration (NASA), United States

***Correspondence:**
Sandro Malacrida
sandromalacrida@gmail.com

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ventilation did not change, suggesting that work efficiency was maintained. They observed a higher prevalence of overweight and obesity, as well as sedentarism in all miner populations: there was an increase in cardiometabolic risk unrelated to altitude, despite those markers of inflammation like hsCRP were increased at moderate altitudes. Fan et al. studied muscular and cerebral tissue oxygenation using near-infrared spectrometry (NIRS) in ten Han Chinese and ten Tibetan subjects during incremental cycling to exhaustion in normobaric normoxia and in hypobaric hypoxia simulating 5,000 m. The results showed a higher brain tissue oxygenation in Tibetan subjects compared to Han Chinese ones during maximal exercise in normoxia, but lower muscle tissue oxygenation during exercise in hypoxia. The authors conclude that Tibetan subjects seem to privilege oxygenation of the brain at the expense of that of the muscle. Functional effects of acute hypobaric hypoxia exposure on the brain were investigated by Falla et al. They reported a reduced processing speed in the first 24 h of exposure of lowlanders at 3,200 m that was quickly reversed after 36 h. These results demonstrate a cognitive impairment after acute exposure to altitude and the burden of an increased risk of accidents. Pernett et al. with an original and proved method studied the effect of 10 minutes acute normobaric hypoxia on spleen volume contraction. They showed a significant spleen volume contraction with Hb increase. This rapid spleen response is evident already after 3 min and can have a protective effect during the first minutes of sudden exposure to severe hypoxia. Sibonama et al. aimed to differentiate subjects suffering from acute mountain sickness (AMS) from those who do not, analyzing urine metabolites with nuclear magnetic resonance (NMR) based metabolomics. In this preliminary report they showed differences in the amount of creatine and acetylcarnitine (elevated), xanthine, hypoxanthine, and taurine (suppressed) in the urine between the subjects suffering from AMS vs. those who do not. They hypothesize that a metabolite profile at sea level could help in the screening for AMS susceptibility at altitude. Khalife et al. aimed to evaluate the possibility to induce erythropoietin (EPO), reticulocytes and hemoglobin stimulation in patients after surgery, avoiding the need for blood transfusions. They investigated the so-called NOP (Normobaric Oxygen Paradox) a physiologic mechanism of relative hypoxia after an exposure to hyperoxia, that induces an increase of endogenous erythropoietin (EPO) production. The comparison between a NOP-treated group and a control group did not show any difference on EPO increase, reticulocytes count and hemoglobin. Antonelli et al. investigated the expression of cytokines IL-6, IL-8, and VEGF (key mediators of the hematopoietic niche) *in vitro* in human macrophages and cell lines under anoxic conditions and after a treatment with oxygenated or deoxygenated red blood cells (RBCs). They found that the treatment with oxygenated RBCs up-regulates IL-8 mRNA, down-regulates IL-6 and VEGF expression in an HIF-1α independent mechanism in anoxic condition. This does not occur when deoxygenated RBCs are used. These are preliminary finding that can stimulate future research.

Despite the heterogeneity of the studies, this special issue highlights the importance of a better understanding of the responses to hypoxia and how our knowledge is still limited after nearly two centuries. An integrative approach between molecular and physiological measures should be fostered in future in in-field and simulated studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Grocott, M., Montgomery, H., and Vercueil, A. (2007). High-altitude Physiology and Pathophysiology: Implications and Relevance for Intensive Care Medicine. *Crit. Care* 11 (1), 203. doi:10.1186/cc5142

West, J. B., and Richalet, J. P. (2013). Denis Jourdanet (1815-1892) and the Early Recognition of the Role of Hypoxia at High Altitude. *Am. J. Physiol. Lung Cell Mol. Physiol.* 305 (5), L333–L340. doi:10.1152/ajplung.00128.2013

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BRIEF RESEARCH REPORT
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Human Red Blood Cells Modulate Cytokine Expression in Monocytes/Macrophages Under Anoxic Conditions

Antonella Antonelli, Emanuele Salvatore Scarpa and Mauro Magnani*

Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy

In the bone marrow (BM) hematopoietic niche, the oxygen tension is usually very low. Such condition affects stem and progenitor cell proliferation and differentiation and, at cellular level regulates hematopoietic growth factors, chemokines and adhesion molecules expression. In turn, these molecules affect the proliferation and maturation of other cellular components of the niche. Due to the complexity of the system we started the *in vitro* investigations of the IL-6, IL-8, TNFα cytokines expression and the vascular endothelial growth factor (VEGF), considered key mediators of the hematopoietic niche, in human macrophages and macrophage cell line. Since in the niche the oxygen availability is mediated by red blood cells (RBCs), we have influenced the anoxic cell cultures by the administration of oxygenated or deoxygenated RBCs (deoxy RBCs). The results reported in this brief paper show that the presence of RBCs up-regulates IL-8 mRNA while IL-6 and VEGF mRNA expression appears down-regulated. This does not occur when deoxy RBCs are used. Moreover, it appears that the administration of RBCs leads to an increase of TNFα expression levels in MonoMac 6 (MM6). Interestingly, the modulation of these factors likely occurs in a hypoxia-inducible factor-1α (HIF-1α) independent manner. Considering the role of oxygen in the hematopoietic niche further studies should explore these preliminary observations in more details.

Keywords: hypoxia, macrophages, red blood cells, pO_2 variation, cytokine expression modulation

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Reviewed by:
Paolo Laveder, University of Padua, Italy
Giulio Ceolotto, University of Padua, Italy

****Correspondence:***
Antonella Antonelli
antonella.antonelli@uniurb.it

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INTRODUCTION

The bone marrow (BM) is a tissue of complex architecture that is organized into a hematopoietic cell compartment and the stroma, which is mainly composed of fibroblasts, adipocytes, nerves, and the BM's vascular system (Morrison and Scadden, 2014; Tamma and Ribatti, 2017). In BM thin-walled sinusoidal vessels are highly specialized capillaries with a discontinuous basement membrane and fenestrations that facilitate trafficking of cells and soluble factors between the blood and the BM compartment. This vasculature provides not only a route for mature hematopoietic cells to the peripheral circulation but also a place where hematopoietic progenitors differentiate and set the stage for full reconstitution of hematopoiesis, which maintains in the peripheral blood a constant level of the different blood cell types and components (erythrocytes granulocytes, platelets, lymphocytes, etc.). Oxygen tension (pO_2) is an important determinant

of hematopoietic stem and progenitor cell (HSPC) proliferation and differentiation. Thus, understanding the impact of the BM architectural organization on pO_2 levels in extravascular hematopoietic tissue is an important biophysical problem. If oxygen concentration in the atmosphere is normally 21%, corresponding to 159 mmHg, in tissues O_2 levels span from 150 to 20–70 mmHg (2.5–9% oxygen), and markedly lower levels (<1% oxygen) have been described in necrotic tissue sites. The condition of reduced oxygen tension is defined as hypoxia. Reduced oxygen tension (pO_2 38 mm Hg, 5% O_2) has been shown to enhance the production of erythroid, megakaryocytic, and granulocytic-monocytic progenitors *in vitro*. In fact, due to the inaccessibility of bone marrow to direct noninvasive oxygen measurements, some authors have used mathematical modeling of pO_2 distributions in the bone marrow and speculated that stem cells are located at the region with very low pO_2 levels (almost anoxic) because this prevents oxygen radicals from damaging these important cells (Chow et al., 2001). Moreover, previous studies have suggested that local oxygen tension determines the location of hematopoietic stem cells (HSCs) in the BM compartment (Parmar et al., 2007; Spencer et al., 2014).

During megakaryopoiesis, megakaryocytes (Mks) differentiate from HSCs and localize in the proximity of the sinusoid blood vessels where they extend long filaments called proplatelets into the blood vessel lumen through the vascular endothelium where platelets, stemming from their terminal ends, are released into the bloodstream by blood shear forces. Despite their critical role in many physiological functions, little is known about the molecular mechanisms involved in platelet production from Mks, or about the pathogenesis of platelet disorders. The characteristics of the environment surrounding Mks play a fundamental role in the regulation of megakaryopoiesis. However, the study of the bone marrow microenvironment *in vivo* has been hampered because of the diffuse three dimensional (3D) nature of its structure and complexity within the bone cavity, especially in humans where invasive approaches are not possible; for this reason, some emerging strategies to obtain information are based on the use of relevant 3D models that offer a scientific approach to mimic and control the physiology of human bone marrow environment within which cells live (Di Buduo et al., 2015; Abbonante et al., 2020). Several authors have contributed in defining niches and mobilization pathways for HSPCs, including the identification of several cell types involved such as osteoblasts, adventitial reticular cells, endothelial cells, monocytic cells, and granulocytic cells and the main factors that anchor HSPCs in the niche and/or induce their quiescence such as vascular cell adhesion molecule (VCAM)-1, CD44, hematopoietic growth factors, e.g., stem cell factor (SCF), chemokines including IL-12 and IL-8 (Richter et al., 2017). A number of cytokines, growth factors, and non-protein metabolites, such as lipids or ions have been demonstrated to play pivotal roles in bone marrow vascular niche regulation (Zhang et al., 2019). Moreover, there is evidence that macrophage cells play an essential role in the maintenance and regulation of BM vascular niche function by secreting large quantities of several molecules with hematopoietic activity, including interleukins that can affect hematopoietic cells. It has been reported that resting macrophages regulate the maturation of Mks and platelet biogenesis releasing mainly IL-8 (D'Atri et al., 2011). It is also known that some sirtuin enzymes, depending by NAD^+ binding can mediate the transcriptional activation of IL-8 and regulate hypoxia-inducible factor-1 α (HIF-1α) stabilization which is routinely used to screen for hypoxia (Wang et al., 1995; Bauer et al., 2012; Di Girolamo et al., 2013; Edatt et al., 2020). Under normoxic conditions, the α subunit of HIF-1 is hydroxylated by prolyl hydroxylases (PHDs), recognized by the protein product of the von-Hippel-Lindau (VHL) gene, ubiquitinated and degraded by the proteasome (Magnani et al., 1994; Huang et al., 1998; Loboda et al., 2012). In hypoxia the PHDs are not active and consequently HIF-1α is not degraded but can translocate to the nucleus, and can dimerize with the β subunit. The heterodimeric transcription factor induces the transcription of genes mediating cellular adaptation to a low oxygen environment. Macrophages in hypoxic condition also respond with increased IL-6 production that can affect both stromal and hematopoietic cells, for example stimulating Mk growth and maturation *in vitro* as well as increasing Mk ploidy (Murdoch et al., 2005; Tanaka et al., 2014). Expression of also VEGF by macrophages is markedly increased by exposure to hypoxia *in vitro* (Tamura et al., 2020). However, the precise role of macrophages in the regulation of human megakaryo/thrombopoiesis is largely unknown.

In the context of the elucidation of the basic mechanisms of intracellular cross-talks between the different cell components of vascular niche we report preliminary data collected during an *in vitro* study performed with an anoxic cell model about the modulation of the expression of specific cytokines.

MATERIALS AND METHODS

Cell Cultures and Reagents

Human monocyte-derived macrophages were prepared from buffy coats provided by healthy adult volunteers at the Blood Transfusion Center of "*S. Maria* della Misericordia" Hospital in Urbino (PU), Italy. All volunteers signed an informed consent form before donation. Macrophages were prepared by density gradient separation using Lymphoprep solution (specific density, 1.077; Axis-Shield PoC AS, Oslo, Norway). Each experiment was performed with macrophages obtained from a single buffy coat (~50 ml) derived from a single donor. The buffy coat was centrifuged at 150 *g* for 15 min at room temperature to eliminate platelets and serum. The pellet was diluted 1:2 with phosphate buffered saline (PBS) and overlaid on Lymphoprep solution (2:1 ratio) and centrifuged at 300 *g* for 30 min. The ring of peripheral blood mononuclear cells (PBMCs), was isolated, washed twice with PBS and resuspended in RPMI medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Essentially, the human macrophages have been obtained from monocytes through their distinguishable ability for plastic adherence. After 24 h in culture in 25 cm^2 flasks (Cell Star, greiner bio-one), at 37°C in a humidified 5% CO_2 atmosphere, non-adherent cells were removed

by rinsing with cell medium in order to isolate adherent monocytes. The culture media was renewed every 2 days and monocyte-derived macrophages were used at 7th day of adherence. Experiments were performed with 1.5 x 10^6 macrophages for each condition. MonoMac 6 (MM6) cells, derived from human acute monocytic leukemia (Ziegler-Heitbrock et al., 1988) were obtained from DMSZ GmbH (Braunschweig, Germany) and cultured in 25 cm^2 flasks at a density of 0.5–1 × 10^6 cells/ml as described in (Palma et al., 2011). Approximately 2.5 × 10^6 MM6 cells were used for each experimental condition. MM6, macrophages and RBCs were exposed to anoxia by using an Hypoxia Incubator Chamber (Chamber for generation of a hypoxic environment for tissue culture, StemCell Technologies) flushed with the appropriate 95% N_2 and 5% CO_2 gas mixture for 15 min to reach anoxia condition and placed at 37°C in a humidified incubator 95% air, 5% CO_2. In our experiment design, MM6 cells were treated for 2, 4, 6, 8, 21, and 24 h and human macrophages for 2, 4, 6, and 24 h. Normoxic cells were also maintained in the incubator as control samples. Moreover, in comparison to anoxic condition, some cell samples were also treated with $CoCl_2$ (Sigma Aldrich), a widely used chemical hypoxia mimetic model; $CoCl_2$ was diluted in complete cell culture media prior to cell stimulation. It has been demonstrated that $CoCl_2$ induces hypoxia-regulated genes by stabilizing HIF-1α in normoxia (Yuan et al., 2003).

The human RBCs were prepared from blood collected in heparinized tubes and derived from healthy adult volunteers of Transfusion Center who signed an informed consent. For all the experiments, blood derived from six donors was used. The blood from a single volunteer was used to prepare RBCs for each experiment. RBCs were isolated as already reported (Antonelli et al., 2020) and resuspended in the same buffer at 10% hematocrit before being deoxygenated or not and administered to anoxic MM6 and human macrophages cells for 3 h. After 3 h of incubation with RBCs, the MM6 cells were packed by centrifugation at 900 *g* and RBCs present in the pellet were lysed with sterile distilled water for 3 min. Immediately afterwards, MM6 cells were resuspended in phosphate buffer saline (PBS) and washed twice by centrifugation to eliminate hemoglobin released from RBCs. Instead, RBCs were removed from adherent human macrophages by three washes with PBS buffer. Total cell extracts obtained using specific lysis buffers were processed for real-time quantitative PCR (RT-qPCR) analysis of IL-6, IL-8, TNFα, VEGF, and hypoxia-inducible factor-1α (HIF-1α) mRNA expression or to detect HIF-1α protein by Western blotting analysis.

Western Blotting Analysis

Cells were lysed in buffer containing Urea 6M, Tiourea 2M, DTT 100 mM, Tris-HCl 30 mM, pH 7.5, Triton 1% and glycerol 9% supplemented with protease inhibitors (cComplete Mini; Roche, Basel, Switzerland); lysates were boiled within 7 min, sonicated twice at 100 Watt for 10 s and cleared by centrifugation at 15,000 × *g* for 10 min, then the supernatants were recovered. The proteins determined by using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, United States) were resolved by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and afterward transferred onto nitrocellulose membrane (100 V, 70 min at 4°C). The blots were probed with the following primary antibodies: anti-HIF-1α (#14179, monoclonal, recognizing amino acidic residues surrounding Lys460 that is codified by the exon 10 of HIF1A CDS[1]) from Cell Signaling Technology (Danvers, MA, United States); anti-Lamin A/C (#sc-376248, monoclonal) from Santa Cruz Biotechnology (Dallas, TX, United States); anti-β-actin (#VMA00048, monoclonal) from Bio-Rad (Hercules, CA, United States). Immunoreactive bands were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad). Peroxidase activity was detected with the enhanced chemiluminescence detection method (WesternBright ECL, Advasta, Menlo Park, CA, United States) using the ChemiDoc MP Imaging System (Bio-Rad). Quantification of the protein bands has been performed using Image Lab analysis software version 5.2.1 (Bio-Rad).

Real-Time Quantitative PCR

Gene-specific expression analyses were performed as already reported (Scarpa et al., 2020). Fluorescence intensity of each amplified sample was measured with an ABI PRISM 7500 Sequence detection system (Applied Biosystems, Foster City, CA, United States). All measurements were performed at least in triplicate and reported as the average values ± standard deviation of the mean (mean ± SD). Target gene values were normalized with B2M mRNA measurements, and expression data were calculated according to the $2^{-\Delta\Delta CT}$ method. Primers were designed using Primer 3 Plus, and their sequences are: IL8-F: 5'-TTGCCAAGGAGTGCTAAAGAA-3'; IL8-R: 5'-GCC CTCTTCAAAAACTTCTCC-3'. IL6-F: 5'-AATTCGGTACATC CTCGACGG-3'; IL6-R: 5'-GGTTGTTTTCTGCCAGTGCC-3'. VEGF-F: 5'-TCACAGGTACAGGGATGAGGACAC-3'; VEGF-R: 5'-CAAAGCACAGCAATGTCCTGAAG-3'. B2M-F: 5'- GCCTG CCGTGTGAACCAT-3'; B2M-R: 5'-CATCTTCAAACCTCCATG ATGCT-3'. TNFα-F: 5'-GCCCAGGCAGTCA-GATCATCTTC-3'; TNFα-R: 5'-TGCCCCTCAGCTT-GAGGGT-3'. HIF1A-F: 5'-TCTG GGTTGAAACTCAAGCAACTG-3'; and HIF1A-R: 5'-CAACCGG TTTAAGGACACATTCTG-3'.

Statistical Analysis

The data were expressed as mean ± SD of three independent experiments. Student's *t*-test and ANOVA test performed with Past Software version 3 were used for statistical analysis of the data; differences between groups were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Human peripheral blood macrophages and MM6 cells (showing functional features of mature blood monocytes), were considered as cell models to study the effect of anoxia exposure on the expression of some biological factors with a regulatory role in the environment of BM vascular niche. Recent studies show

[1]https://www.ncbi.nlm.nih.gov/nuccore/NM_001530.4

that macrophages respond rapidly to the hypoxia condition by altering their expression of a wide array of genes. Among the various roles played by macrophages, they are also responsible for regulating tissue oxygenation by influencing the formation of new blood vessels and modulating vascular permeability. Notably, in response to hypoxia, macrophages have been shown to induce proangiogenic molecules such as VEGF, and IL-8. Some studies have already shown that HIFs, but not NF-kB, are important transcriptional effectors regulating the responses of macrophages exposed to 18 h hypoxia (Fang et al., 2009). It is currently accepted that resting macrophages, which are relevant components of the BM stroma, release soluble factors that promote megakaryocyte growth, proplatelet production, and platelet release (D'Atri et al., 2011). Here, we investigated the cellular response to anoxia stimulus obtained by using a hypoxic incubator chamber and a gas mixture of 95%N_2 and 5%CO_2. Firstly, the results were compared to those obtained with the chemical agent $CoCl_2$ which has been shown to mimic the hypoxic conditions in cells by stabilizing the transcription factor HIF-1α. When $CoCl_2$ is added to the cell culture, the hydroxylation activity of PHDs is inhibited, therefore HIF-1α protein is not degraded through the ubiquitin/proteasome pathway (Munoz-Sànchez and Chanez-Càrdenas, 2019). **Figure 1** shows a representative time-course experiment performed with MM6 cell model exposed to anoxia (**Figures 1A–C**) and to 100 μM $CoCl_2$ (**Figures 1D–F**). When $CoCl_2$ is added to the cell culture, the hydroxylation activity of PHDs is inhibited, therefore HIF-1α protein is not degraded through the ubiquitin/proteasome pathway. mRNA levels of some cytokines such as IL-8, IL-6, and VEGF have been measured. Cells maintained in normoxia (21% O_2) for the same time period of treatment were used as controls. Under anoxia, an increase of IL-8 mRNA levels (**Figure 1A**) was found within the 24 h of experimental design; IL-8 expression levels reach values that are about 7-folds higher than controls; the highest expression values are at 4 and 24 h of incubation (4 h: 3.21 ± 0.93, $p = 0.004$; 24 h: 7.62 ± 2.24, $p = 0.015$) similarly to what happens with the $CoCl_2$ treatment (**Figure 1D**) performed in the same time range (4 h: 6.81 ± 1.41, $p = 0.025$; 24 h: 9.10 ± 3.64, $p = 0.018$). Moreover, IL-6 expression levels significantly increase after 24 h of anoxia exposure (4.81 ± 0.70, $p = 0.029$; **Figure 1B**), whereas in $CoCl_2$-treated cells the IL-6 levels increase already from 2 h with a peak of expression at 4 h (15.32 ± 0.44, $p < 0.001$), **Figure 1E**. Furthermore, VEGF expression levels remarkably increase only after 21 h and 24 h anoxia treatment (**Figure 1C**), with a peak at 21 h (5.80 ± 1.10, $p = 0.003$) and also after $CoCl_2$ treatment (**Figure 1F**), with a maximum peak at 8 h (2.90 ± 0.37, $p = 0.046$). At the same time, we have evaluated by western blot analyses the content of HIF-1α protein in cell lysates of MM6 cells exposed to anoxia (**Figure 1G**) and $CoCl_2$ (**Figure 1H**). It is evident that HIF-1α expression was remarkably induced under anoxic condition after 6 h of exposure with an increase of the protein levels up to 21 h followed by a return toward basal levels at 24 h (**Figure 1G, Table 1**). Similar results obtained after CoCl2 treatment are shown in **Figure 1H** where the HIF-1α protein appears in different isoforms (in the range of 75–100 kDa), as already described in literature (Monsef et al., 2010), **Figure 1H** and **Table 1**. In fact, it is not uncommon to detect protein bands other than at the one expected at 120 kDa; different forms of HIF-1α protein detectable after $CoCl_2$ treatment, have been already described (Rana et al., 2019).

The same experimental design was performed with monocyte-derived macrophages isolated from human peripheral blood. **Figure 2** shows a different response to the anoxia or $CoCl_2$ stimuli with respect to leukemia MM6 cells. After 6 h of anoxia exposure the IL-8 mRNA level increases of about 4-fold (4.02 ± 1.16, $p = 0.047$) respect to normoxia value (**Figure 2A**) whereas with $CoCl_2$ treatment the maximum expression occurs at shorter times (2 h; 4.43 ± 1.40, $p = 0.003$; **Figure 2D**); in both cases the mRNA expression decreases within the 21 h. IL-6 mRNA expression seems to augment after already 2 h of $CoCl_2$ (**Figure 2E**) or anoxia (**Figure 2B**) treatment reaching values of about 1.5-fold (1.76 ± 0.53, $p = 0.603$) and 4-fold (3.86 ± 1.05, $p = 0.041$) increase, respectively. VEGF expression appears to have a similar trend when macrophages are exposed to both stimuli (**Figures 2C,F**) which induce a remarkable increase of mRNA levels in the range of 4–6 h of treatment (peak at 4 h for anoxia; 8.49 ± 0.05, $p < 0.001$; peak at 6 h for $CoCl_2$, 5.74 ± 1.59, $p < 0.001$).

The results obtained from these analyses are in accordance with literature; for example, it is well known that human macrophages respond to oxygen stress such as an anoxia exposure with augmented production of IL-8 (Metinko et al., 1992; Rydberg et al., 2003).

Western blotting analysis of HIF-1α protein in the macrophages extracts obtained after anoxia (**Figure 2G**) and $CoCl_2$ (**Figure 2H**) stimuli at the indicated times showed increased amounts of the protein, as 200 kDa band, already from the 2 h of treatment (**Table 1**). HIF-1α protein can be still detected at 24 h after anoxia exposure while it decreases at the same time after $CoCl_2$ treatment. After the evaluation of cell response to anoxia stimulus establishing the highest peaks of gene expression of these specific cytokines (6 h for human macrophages and 21 h for MM6 cells), we have *in vitro* studied the impact of RBCs on these cell lines in order to understand if a variation of pO_2 level in anoxic environment could alter or regulate the expression of these soluble factors. In fact, oxygen transport to the hematopoietic niche is mediated by RBCs and RBC oxygenation represents more closely the niche oxygen availability rather than the simple oxygen concentration in the tissue cultures.

Figure 3 shows the IL-8, IL-6, VEGF and TNFα mRNA levels obtained after the addition of RBCs at 10% hematocrit for 3 h to MM6 (**Figures 3A–D**) and human macrophage cells (**Figures 3E–H**), respectively. The data were compared with results obtained after the administration of deoxy RBCs to MM6 cells or human macrophages incubated at the same condition of cells treated with native RBCs. It is evident that a further increase in IL-8 expression levels in anoxic MM6 cells incubated with native RBCs occurs (anoxia 21 h + RBCs, 14.08 ± 2.93 vs. anoxia 21 h; 5.88 ± 1.92, $p = 0.048$, **Figure 3A**), but this treatment did not lead to an increase in IL-6 expression levels, rather to a decrease (anoxia 21 h + RBCs 1.565 ± 0.62 vs. anoxia 21 h, 2.84 ± 0.75, $p = 0.48$, **Figure 3B**). Moreover, VEGF mRNA levels significantly decrease (anoxia 21 h + RBCs

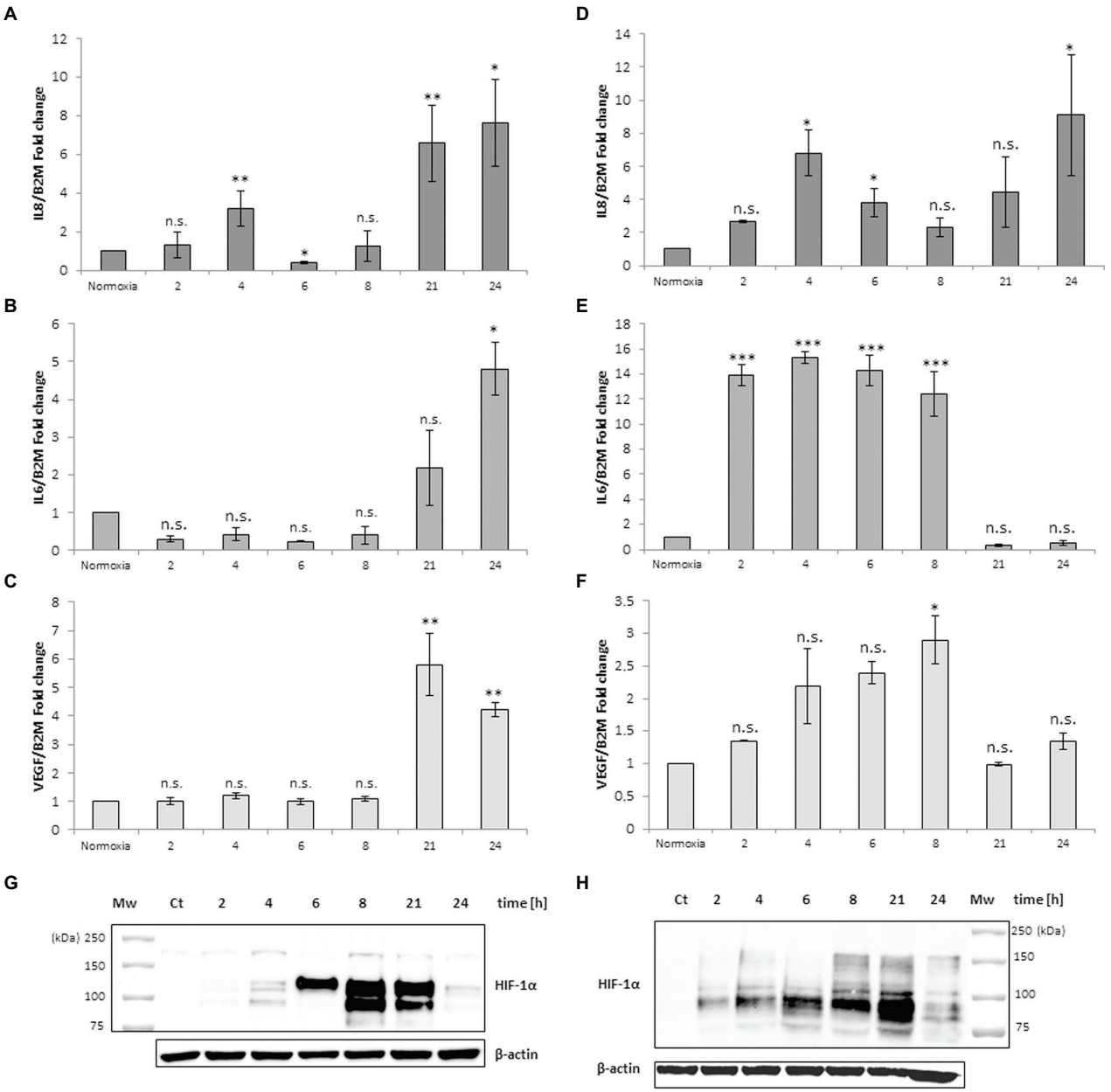

FIGURE 1 | mRNA levels of IL-8, IL-6, and vascular endothelial growth factor (VEGF) in cell extracts of MonoMac 6 (MM6) cells after anoxia (95% N_2, 5%CO_2; **A–C**) and 100 μM $CoCl_2$ **(D–F)** treatment at different times (hours). Values are expressed as mean ± SD; $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant. Western blotting analysis shows the hypoxia-inducible factor-1α (HIF-1α) protein in total extracts after anoxia **(G)** and $CoCl_2$ **(H)** treatment of MM6 cells at the indicated times.

1.525 ± 0.28 vs. anoxia 21 h 3.01 ± 0.35, $p = 0.004$, **Figure 3C**). In addition, the incubation of MM6 cells with deoxy RBCs did not lead to an increase of IL-6 and VEGF expression levels (for IL-6; anoxia 21 h + deoxy RBCs 3.52 ± 0.11 vs. anoxia 21 h, 2.84 ± 0.75, $p = 0.56$). Concerning VEGF mRNA levels, the values decreased when deoxy RBCs were administered to anoxic MM6 (anoxia 21 h + deoxy RBC 1.19 ± 0.18 vs. anoxia 21 h, 3.01 ± 0.35, $p = 0.007$) as also after reoxygenation of anoxic cells (anoxia 21 h + air, 1.05 ± 0.15 vs. anoxia 21 h, 3.01 ± 0.35, $p = 0.039$, **Figure 3C**). In addition, IL-8 mRNA levels appear also decreased after the treatment with deoxy RBCs compared to values obtained with anoxia exposure (anoxia 21 h + deoxy RBCs 1.23 ± 0.36 vs. anoxia 21 h 5.88 ± 1.92, $p = 0.037$). Moreover, the value of IL-8 mRNA level of anoxic MM6 cells treated with deoxy RBCs was lower than value of cells treated with native RBCs (anoxia 21 h + RBCs

TABLE 1 | Quantification of the HIF-1α protein bands in the immunoblots reported in **Figures 1**–**3**.

Figure 1G	**Net Intensity (A.U.)**	**SD**	***p* value**	**Figure 1H**	**Net intensity (A.U.)**	**SD**	***p* value**	**Figure 3I**	**Net intensity (A.U.)**	**SD**	***p* value**
Control	0.0016	0.0004		Control	0.0006	0.0001		Control	0.0246	0.0071	
Anoxia 2 h	0.0778	0.0114	0.294	$CoCl_2$ 2 h	0.0307	0.0073	0.002**	Anoxia 21 h	0.2047	0.0570	0.025*
Anoxia 4 h	0.3220	0.1207	0.002**	$CoCl_2$ 4 h	0.0810	0.0250	0.005**	Anoxia + RBCs	0.0544	0.0236	0.090
Anoxia 6 h	3.7122	1.4873	0.006**	$CoCl_2$ 6 h	0.0897	0.0250	0.003**	Anoxia + RBCs deox	0.1095	0.0349	0.034*
Anoxia 8 h	6.0074	2.7509	0.008**	$CoCl_2$ 8 h	0.1323	0.0443	0.007**	Anoxia + Air	0.0231	0.0121	0.648
Anoxia 21 h	3.1870	1.4013	0.006**	$CoCl_2$ 21 h	0.2883	0.1287	0.003**	$CoCl_2$	0.4851	0.1962	0.027*
Anoxia 24 h	0.1643	0.0543	0.181	$CoCl_2$ 24 h	0.0303	0.0128	0.016*	**Figure 3J**	**Net intensity (A.U.)**	**SD**	**p value**
Figure 2G	**Net intensity (A.U.)**	**SD**	**p value**	**Figure 2H**	**Net intensity (A.U.)**	**SD**	**p value**	Control	0.0011	0.0004	
Control	0.0065	0.0022		Control	0.1348	0.0356		Anoxia + Air	0.0017	0.0011	0.297
Anoxia 2 h	0.0173	0.0070	0.004**	$CoCl_2$ 2 h	0.9576	0.1652	<0.001***	Anoxia 6 h	0.1505	0.0374	0.020*
Anoxia 4 h	0.0458	0.0255	0.024*	$CoCl_2$ 4 h	1.0009	0.0931	<0.001***	Anoxia + RBCs	0.0062	0.0006	0.061
Anoxia 6 h	0.0150	0.0078	0.146	$CoCl_2$ 6 h	0.4786	0.1036	0.005**	Anoxia + RBCs deox	0.0398	0.0169	0.026*
Anoxia 24 h	0.0199	0.0084	0.004**	$CoCl_2$ 24 h	0.2218	0.0652	0.112	$CoCl_2$	0.8187	0.2710	0.035*

The quantification of HIF-1 protein was performed by densitometry measurements using Image Lab software version 5.2.1 (Bio-Rad). **Figures 1G,H, 3I** *refer to MM6 cells and* **Figures 2G,H, 3J** *to human macrophages. Statistical analyses were performed with Past 3 Software, using ANOVA method for data of* **Figures 1G,H, 2G,H** *and paired student's T-test for* **Figures 3I,J**. *A.U., arbitrary units.*

**p < 0.05.*

***p < 0.01.*

****p < 0.001.*

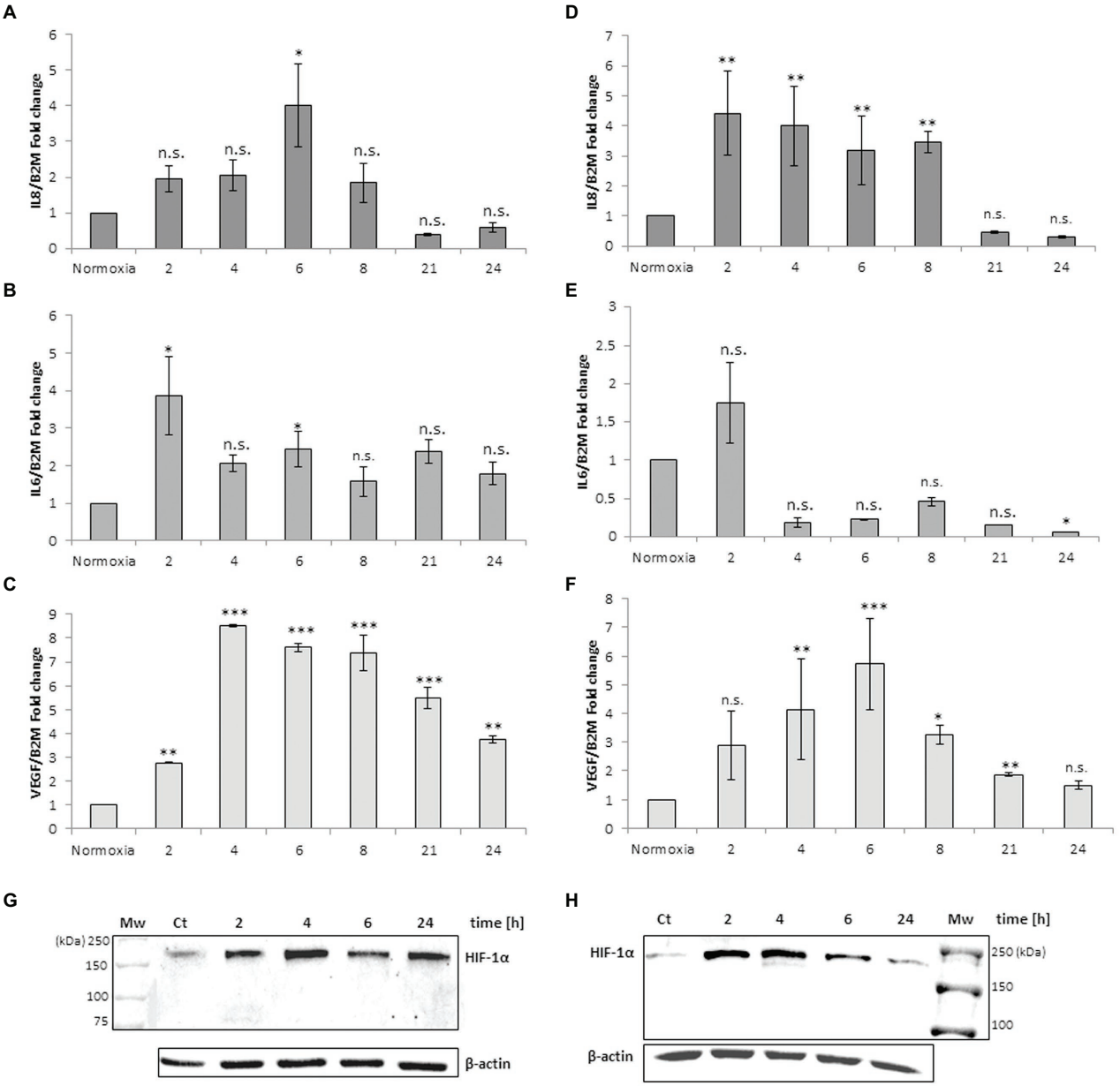

FIGURE 2 | mRNA levels of IL-8, IL-6 and VEGF in cell extracts of human macrophages from peripheral blood after anoxia (95% N_2, 5%CO_2; **A–C**) and 100 μM $CoCl_2$ **(D–F)** treatment at different times (hours). Values are expressed as mean ± SD; $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. not significant. Western blotting analysis shows the HIF-1α protein in total extracts after anoxia **(G)** and $CoCl_2$ **(H)** treatment of human macrophages at the indicated times.

14.08 ± 2.93, $p = 0.047$), **Figure 3A**. Interestingly, **Figure 3I** that relates to western blot analysis of the same cell samples, shows a strong decrease of HIF-1α band protein when anoxic MM6 cells are treated with native RBCs (lane 3), similarly to reoxygenated MM6 cells (lane 5), while the protein band is evident after 21 h anoxia exposure (lane 2) or after 100 μM $CoCl_2$ treatment (lane 6), as expected. The administration of deoxygenated RBCs to anoxic MM6 cells does not seem to lead to the same result, since HIF-1α protein band is maintained (lane 4). Densitometric analysis of **Figure 3I** has been reported in **Table 1**. In **Figure 3I** the blot of lamin protein as control protein was reported in addition to actin, in order to show that the protein content derived only from MM6 cells and not from RBCs. **Figure 3** shows in similar way the levels of IL-8, IL-6, TNFα and VEGF mRNA expression in human macrophages as response to the same stimuli; IL-8 mRNA level appears to increase in anoxic cells treated with native RBCs (anoxia 6 h + RBCs, 3.62 ± 0.11-folds vs. anoxia 6 h,

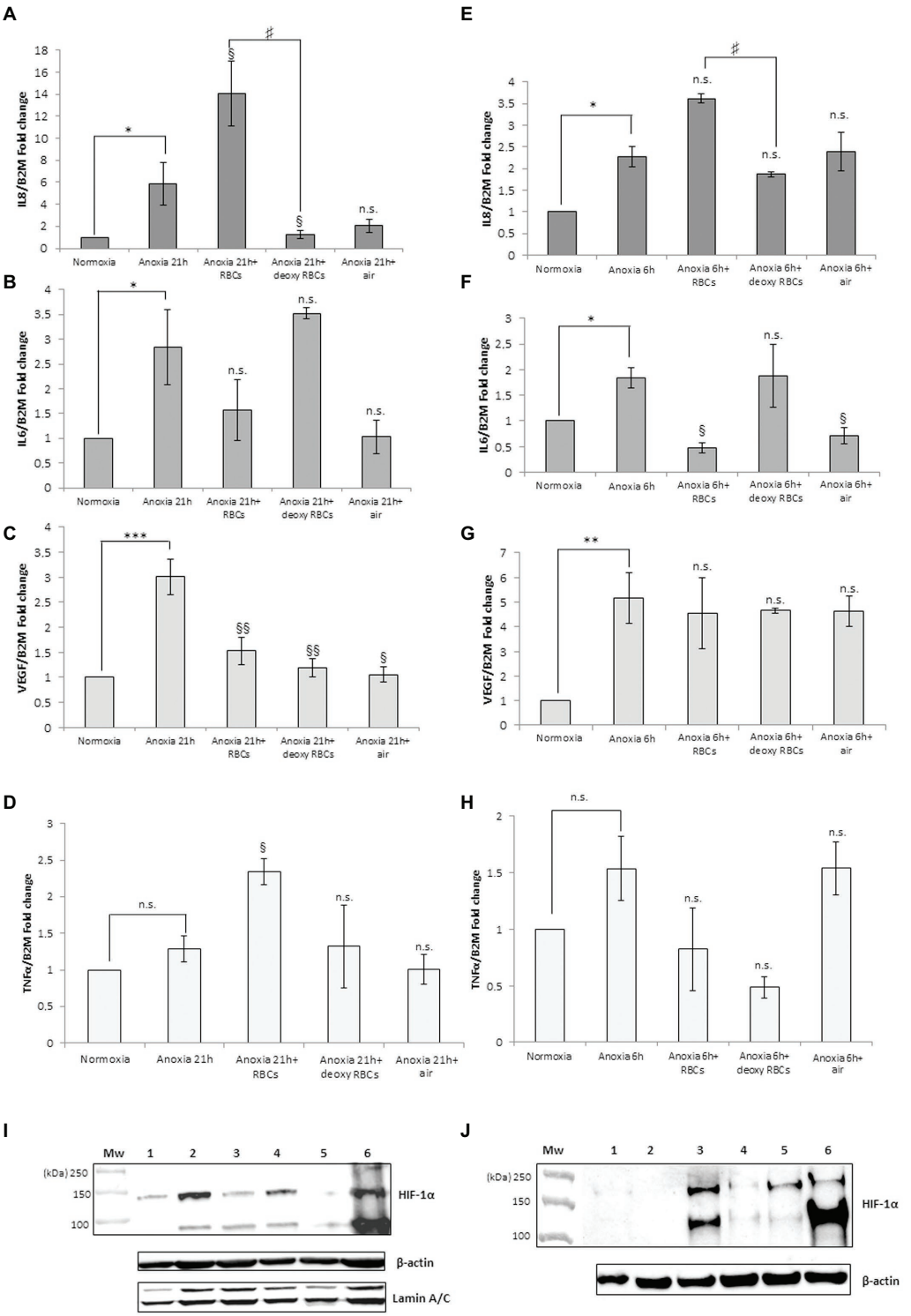

FIGURE 3 | mRNA levels of IL-8, IL-6, VEGF and TNFα in cell extracts of MM6 cells **(A–D)** and human macrophages **(E–H)** after the incubation with red blood cells (RBCs; 10% Ht) deoxygenated or not. Values are expressed as mean ± SD; $n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with normoxic samples. $^{§}p < 0.05$, $^{§§}p < 0.01$ when compared with anoxic samples. $^{\#}p < 0.05$ when anoxia + RBCs samples were compared with anoxia + deoxy RBCs samples. Western blotting analysis shows the HIF-1α protein in total extracts of MM6 cells (**I**, 1. Control 2. Anoxia 21 h 3. Anoxia 21 h + RBCs 3 h 4. Anoxia 21 h + deoxy RBCs 3 h 5. Anoxia 21 h + air 3 h; and 6. 100 μM $CoCl_2$ 21 h) or human macrophages (**J**, 1. Control 2. Anoxia 6 h + air 3 h 3. Anoxia 6 h 4. Anoxia 6 h + RBCs 3 h 5. Anoxia 6 h + deoxy RBCs 3 h; and 6. 100 μM $CoCl_2$ 6 h) after incubation with RBCs (10% Ht) deoxygenated or not.

2.27 ± 0.24, $p = 0.61$, **Figure 3E**) whereas IL-6 levels significantly decrease (anoxia 6 h + RBCs 0.47 ± 0.10-folds vs. 1.84 ± 0.20, $p = 0.045$; **Figure 3F**). Moreover, VEGF mRNA levels do not seem to change (4.54 ± 1.43 vs. 5.16 ± 1.04, $p = 0.56$), **Figure 3G**. On the contrary, the treatment with deoxy RBCs results in the expression levels of the target genes which do not differ from those found in the cells exposed to only anoxia. In fact, no significant increase of neither IL-8 (**Figure 3E**) nor VEGF (**Figure 3G**) mRNA values were found respect to those found in the anoxic cells (for IL-8; anoxia 6 h + deoxy RBC, 1.87 ± 0.06 vs. anoxia 6 h 2.27 ± 0.24, $p = 0.29$; for VEGF; 4.65 ± 0.10 vs. anoxia 6 h 5.16 ± 1.04, $p = 0.26$). Moreover, IL-6 mRNA expression after anoxia exposure (**Figure 3F**) significantly decreases after the re-oxygenation of cells, and it does not change after incubation with deoxy RBCs (anoxia 6 h + air 0.71 ± 0.16 and anoxia 6 h + deoxy RBCs 1.87 ± 0.62 vs. anoxia 6 h 1.84 ± 0.20, with $p = 0.013$ and $p = 0.88$, respectively). In addition, the increase of IL-8 mRNA value (**Figure 3E**) after administration of native RBCs is significantly different respect to value obtained with deoxy RBCs (anoxia 6 h + RBCs 3.62 ± 0.11 vs. anoxia 6 h + RBC deoxy 1.87 ± 0.06, $p = 0.042$). Furthermore, it appears that anoxia treatment leads to slight increase of TNFα mRNA levels in both MM6 (1.29 ± 0.18-fold) and human macrophages (1.54 ± 0.28-fold) respectively, **Figures 3D,H**. Moreover, the administration of native RBCs seems to further significantly increase these values (2.34 ± 0.17-fold, $p = 0.042$) in anoxic MM6 cells, while the ddeoxy RBCs do not (1.32 ± 0.57-fold, $p = 0.756$), **Figure 3D**. Instead, the treatments with both native and deoxy RBCs to anoxic human macrophages leads to a not significant decrease of TNFα expression levels (0.82 ± 0.36-fold and 0.48 ± 0.09-fold, respectively), **Figure 3H**. Although increased levels of TNFα mRNA after RBCs treatment were evidenced, they did not reach values obtained for IL-8 cytokine; however, the data could be interesting considering that it is known that TNFα is the principal cytokine driving the adhesion of MM6 cells to endhotelial cells as already reported (Schuettpelz and Link, 2013; Poussin et al., 2014). The **Supplementary Tables 1, 2** report the RTqPCR data of **Figure 3**.

Figure 3J, showing analysis of HIF-1α protein in human macrophage samples, evidences a response to these treatments similar to MM6 cells. In fact, the incubation of macrophages exposed 6 h to anoxia with native RBCs leads to the disappearance of HIF-1α in total cell extracts (lane 4) and this partially occurs with the deoxy RBCs (lane 5). Densitometric analysis of **Figure 3J** has been reported in **Table 1**. The presence of different HIF-1α protein isoforms revealed by western blotting analysis (**Figures 1G,H**; **Figures 2G,H**; **Figures 3I,J**) could be explained by their possible post-translational modifications. It is not unusual to find in literature several articles concerning the presence of a broad range of HIF-1α isoforms, commonly reported in both healthy and tumor cells (Monsef et al., 2010). Several studies report on HIF-1α isoforms lacking several exons than the wild type full length isoform. Some of these isoforms encode cytoplasmic HIF-1α protein or proteins with altered transcriptional activity compared to the wild type protein (Gothié et al., 2000; Chun et al., 2001, 2002; Depping et al., 2004; Lukashev and Sitkovsky, 2008). Different HIF-1α isoforms, such as HIF-1α 1.2 which is 59 amino acids shorter (86 KDa) than wild type HIF-1α (93 KDa; Depping et al., 2004), HIF1α 1.3 which encodes a functional protein of 95 KDa (Lukashev and Sitkovsky, 2008), and isoforms lacking either exon 12 (62 KDa) or exon 14 (82 KDa; Gothié et al., 2000; Chun et al., 2001) or exons 11 and 12 (58 KDa; Chun et al., 2002) were found. Importantly, the anti-HIF-1α antibody used in our analyses can recognize also the HIF-1α protein isoforms codified by the alternative splicing variants that act as dominant negative HIF-1α isoforms (Chun et al., 2001, 2002), which are able to inhibit the activity of wild type full length HIF-1α. The different molecular weights showed by our immunoblots could be explained by both alternative splicing events or by different post-translational modifications including phosphorylation, S-nitrosylation and acetylation (Toffoli et al., 2007; Geng et al., 2012; Sanhueza et al., 2020). Moreover, the dimeric protein that appears at a position of approximately 200 kDa could be explained by the presence of HIF-1 α complexed with other proteins or factors (e.g., the constitutively expressed HIF-1β-subunit, or enzymes such as VHL).

Our data indicate that RBCs could up-regulate IL-8 mRNA and down-regulate IL-6 mRNA and VEGF mRNA expression in a way that is independent of HIF-1α in human macrophages in anoxic condition; similarly, this occurs also for the human monocytic MM6 cells that respond also with an increased TNFα mRNA expression.

Moreover, we investigated if the expression of HIF-1 α mRNA levels could be changed in anoxic MM6 and macrophage cells treated with RBCs to provide another way supporting the not involvement of HIF-1α in the up-regulation of IL-8. These results are reported in **Supplementary Figure 1**, showing a decrease of HIF-1α mRNA levels in cell extracts of human MM6 and macrophage anoxic cells treated with native and deoxygenated RBCs; specifically, for MM6 cells 0.57 ± 0.29-fold ($p = 0.157$, **Supplementary Figure 1A**) and for macrophages 0.75 ± 0.14-fold ($p = 0.115$, **Supplementary Figure 1B**) when native human RBCs were administered. When deoxygenated RBCs were administered to MM6 cells, 0.79 ± 0.12-fold ($p = 0.102$, **Supplementary Figure 1A**) and to human macrophages 0.84 ± 0.21-fold ($p = 0.295$, **Supplementary Figure 1B**) were found. Thus, HIF-1α mRNA levels were not significantly different when compared with levels of anoxic cells (MM6 cells 0.62 ± 0.21-fold, **Supplementary Figure 1A**; human macrophages 0.78 ± 0.05-fold, **Supplementary Figure 1B**). Since both HIF-1α mRNA and corresponding protein levels decrease in cells treated with native RBCs, we can hypothesize that HIF-1α transcription factor could not be directly involved in the increase of IL-8 expression levels. In literature, some works reported evidences that link the upregulation of IL-8 with the increased recruitment of some transcriptional factors binding IL-8 promoter, like Egr-1 (Singha et al., 2014). Probably, the different expression peaks of IL-8 mRNA in MM6 (21 h) and macrophage cells (6 h) could be explained by the fact that Egr-1 or other

transcription factors accumulate early in macrophages after exposure to hypoxic/anoxic conditions and lately in monocytic leukemia cells (Elbarghati et al., 2008).

We think that RBCs, as modulator of pO_2, could interact with these cell systems through mechanisms that would be interesting to study.

Since our data indicate that native RBCs administrated to monocyte-macrophage cell lines induce a specific modulation of IL-8 mRNA expression, it could be useful to understand which are the transcription factors on which these specific cellular responses depend. This aspect is important considering the putative role that this interleukin plays in vascular niche of BM. It was shown that, as other soluble factors, the IL-8 has a pleiotropic role and affects, directly or indirectly, different cellular pathways such as hematopoietic differentiation, cell survival and angiogenesis (Dudek et al., 2003; Aronovich et al., 2013; Poulos et al., 2013). On the other hand, it is known that the maturation of RBCs involves some interactions with macrophages, first during their development in the BM, later in the blood stream with macrophages found in the liver and spleen. These interactions are essential to maintain RBC homeostasis or to ensure the correct removal of aged or damaged RBCs. Our studies could aid to reveal how some biological factors, derived from macrophage and RBC interactions, play a role in BM vascular niche. Considering that contacts take place between macrophage and erythroblastic islands and that erythrocytic cells are capable of migrating toward BM sinusoids as erythroid precursors, it is also reasonable to think that the RBC-macrophages interactions can affect or regulate the function of the other cell components in bone vascular niche, such as megakaryocytic cells associated with the BM vasculature (Machlus and Italiano, 2013). Several researchers are attempting to clarify these aspects studying the dynamic interactions between cellular and molecular components of the BM vascular niche (Zhang et al., 2019); co-culture cell models, where different cells types (for example erythrocytes, macrophages, megakaryocytes and endothelial cells) coexist together, represent versatile tools for investigating these cellular interactions. For example, the biocompatible bioengineered tissue-models, such as 3D BM device mimicking the different features of the BM environment (Di Buduo et al., 2015; Abbonante et al., 2020) are promising approaches to study megakaryocyte function; once in contact with the biomaterial, megakaryocytes extend proplatelets into the perfused culture medium, mimicking blood shear stress. Therefore, the possibility to take advantage of this perfusion bioreactor chamber mimicking *ex vivo* the vascular niche, will allow to transfer in a 3D cell model the results herein reported in order to better understand how the RBCs can influence the intercellular cross-talk in the vascular niche.

CONCLUSION

Herein, we investigated the modulation of mRNA expression of few key cytokines (IL-6, IL-8, VEGF, and TNFα) and the possible role of HIF-1α transcription factor, in the early response to the stimuli considered in our experimental cell model; the data indicated the not involvement of HIF-1α in the regulation of these specific cytokines. HIF-1 independent cellular pathways have been reported in solid tumor such as the glioblastoma (Tardòn et al., 2020). Other mechanisms that do not involve HIF-1α have already been described, thus elucidating a novel HIF-independent point of control of cellular metabolism, energetics, and post-transcriptional gene regulations by O_2, such as mTOR inactivation, or the activation of NF-kB through reactive oxygen species (ROS; Arsham et al., 2003; Mizukami et al., 2005, 2007; Lluis et al., 2007; Park et al., 2012). We can speculate that the treatment of anoxic cells with RBCs leads to a remarkable decrease of HIF-1α, which acts as a repressor of IL-8 (Loboda et al., 2012). This point would explain the observed increase of IL-8 mRNA levels. Furthermore, it was evidenced that, in a model of human glioblastoma, the hypoxia-induced accumulation of HIF-1α was correlated with an increase of IL-6 levels (Xue et al., 2016). Therefore, in our cell model the RBC-mediated decrease of HIF-1α could explain the decrease of IL-6 mRNA levels. Other factors such as HIF-2α, HIF-3α, AP-1, C/EBP and NF-kB may play a role in hypoxia (Shih and Claffey, 1998; Hirani et al., 2001; Rius et al., 2008; Palazon et al., 2014), while ATF-4 and Egr-1 are hypoxia responsive factors in macrophages, but only after early exposures (Elbarghati et al., 2008). In conclusion, a number of transcription factors work together in a tightly regulated fashion to control macrophage activities in hypoxic condition. Further studies are necessary to examine the role of those players under the experimental conditions investigated.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

ETHICS STATEMENT

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. In addition, for investigations involving the use of human blood, an informed consent has been obtained from the subjects involved, on the basis of official document of accordance with the Transfusion Center of "*S. Maria* della Misericordia" Hospital in Urbino (PU), Italy.

AUTHOR CONTRIBUTIONS

AA and MM defined the study and planned the experiments. AA and ES performed experiments and data acquisition and analysis. AA wrote the manuscript. MM revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.632682/full#supplementary-material

REFERENCES

Abbonante, V., Di Buduo, C. A., Malara, A., Laurent, P. A., and Balduini, A. (2020). Mechanisms of platelet release: in vivo studies and in vitro modeling. *Platelets* 31, 717–723. doi: 10.1080/09537104.2020.1774532

Antonelli, A., Szwargulski, P., Scarpa, E. S., Thieben, F., Cordula, G., Ambrosi, G., et al. (2020). Development of long circulating magnetic particle imaging tracers: use of novel magnetic nanoparticles and entrapment into human erythrocytes. *Nanomedicine* 15, 739–753. doi: 10.2217/nnm-2019-0449

Aronovich, A., Nur, Y., Shezen, E., Rosen, C., Klionsky, Y. Z., Milman, I., et al. (2013). A novel role for factor VIII and thrombin/PAR1 in regulating hematopoiesis and its interplay with the bone structure. *Blood* 122, 2562–2571. doi: 10.1182/blood-2012-08-447458

Arsham, A. M., Howell, J. J., and Simon, M. C. (2003). A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of Rapamycin and its targets. *J. Biol. Chem.* 278, 29655–29660. doi: 10.1074/jbc.M212770200

Bauer, I., Grozio, A., Lasigliè, D., Basile, G., Sturla, L., Magnone, M., et al. (2012). The NAD^+-dependent histone deacetylase SIRT6 promotes cytokine production and migration in pancreatic cancer cells by regulating Ca^{2+} responses. *J. Biol. Chem.* 288, 40924–40937. doi: 10.1074/jbc.M112.405837

Chow, D. C., Wenning, L. A., Miller, W. M., and Papoutsakis, E. T. (2001). Modeling pO_2 distributions in the bone marrow hematopoietic compartment. I. Krogh's model. *Biophys. J.* 81, 675–684. doi: 10.1016/S0006-3495(01)75732-3

Chun, Y. S., Choi, E., Kim, T. Y., Kim, M. S., and Park, J. W. (2002). A dominant-negative isoform lacking exons 11 and 12 of the human hypoxia-inducible factor-1alpha gene. *Biochem. J.* 362, 71–79. doi: 10.1042/0264-6021:3620071

Chun, Y. S., Choi, E., Yeo, E. J., Lee, J. H., Kim, M. S., and Park, J. W. (2001). A new HIF-1 alpha variant induced by zinc ion suppresses HIF-1-mediated hypoxic responses. *J. Cell Sci.* 114, 4051–4061.

D'Atri, L. P., Pozner, R. G., Nahmod, K. A., Landoni, V. I., Isturiz, M., Negrotto, S., et al. (2011). Paracrine regulation of megakaryo/thrombopoiesis by macrophages. *Exp. Hematol.* 39, 763–772. doi: 10.1016/j.exphem.2011.03.009

Depping, R., Hägele, S., Wagner, K. F., Wiesner, R. J., Camenisch, G., Wenger, R. H., et al. (2004). A dominant-negative isoform of hypoxia-inducible factor-1 alpha specifically expressed in human testis. *Biol. Reprod.* 71, 331–339. doi: 10.1095/biolreprod.104.027797

Di Buduo, C. A., Wray, L. S., Tozzi, L., Malara, A., Chen, Y., Ghezzi, C. E., et al. (2015). Programmable 3D silk bone marrow niche for platelet generation ex vivo and modeling of megakaryopoiesis pathologies. *Blood* 125, 2254–2264. doi: 10.1182/blood-2014-08-595561

Di Girolamo, M., Fabrizio, G., Scarpa, E. S., and Di Paola, S. (2013). NAD^+-dependent enzymes at the endoplasmic reticulum. *Curr. Top. Med. Chem.* 13, 3001–3010. doi: 10.2174/15680266113136660214

Dudek, A. Z., Nesmelova, I., Mayo, K., Verfaillie, C. M., Pitchford, S., and Slungaard, A. (2003). Platelet factor 4 promotes adhesion of hematopoietic progenitor cells and binds IL-8: novel mechanisms for modulation of hematopoiesis. *Blood* 101, 4687–4694. doi: 10.1182/blood-2002-08-2363

Edatt, L., Poyyakkara, A., Raji, G. R., Ramachandran, V., Shankar, S. S., and Kumar, S. V. B. (2020). Role of sirtuins in tumor angiogenesis. *Front. Oncol.* 9:1516. doi: 10.3389/fonc.2019.01516

Elbarghati, L., Murdoch, C., and Lewis, C. E. (2008). Effects of hypoxia on transcription factor expression in human monocytes and macrophages. *Immunobiology* 213, 899–908. doi: 10.1016/j.imbio.2008.07.016

Fang, H. Y., Hughes, R., Murdoch, C., Coffelt, S. B., Biswas, S. K., Harris, A. L., et al. (2009). Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood* 114, 4–33. doi: 10.1182/blood-2008-12-195941

Geng, H., Liu, Q., Xue, C., David, L. L., Berer, T. M., Thomas, G. V., et al. (2012). HIF1 protein stability is increased by acetylation at lysine 709. *J. Biol. Chem.* 287, 35496–35505. doi: 10.1074/jbc.M112.400697

Gothié, E., Richard, D. E., Berra, E., Pagès, G., and Pouyssegur, J. (2000). Identification of alternative spliced variants of human hypoxia-inducible factor-1alpha. *J. Biol. Chem.* 275, 6922–6927. doi: 10.1074/jbc.275.10.6922

Hirani, N., Antonicelli, F., Strieter, R. M., Wiesener, M. S., Ratcliffe, P. J., Haslett, C., et al. (2001). The regulation of Interleukin-8 by hypoxia in human macrophages-a potential role in the pathogenesis of the acute respiratory distress syndrome (ARDS). *Mol. Med.* 7, 685–697.

Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. U. S. A.* 95, 7987–7992.

Lluis, J. M., Buricchi, F., Chiarugi, P., Morales, A., and Fernandez-Checa, J. C. (2007). Dual role of mitochondrial reactive oxygen species in hypoxia signaling: activation of nuclear factor-{kappa}B via c-SRC and oxidant-dependent cell death. *Cancer Res.* 67, 7368–7377. doi: 10.1158/0008-5472.CAN-07-0515

Loboda, A., Jozkowicz, A., and Dulak, J. (2012). HIF-1 versus HIF-2 – is one more important than the other? *Vasc. Pharmacol.* 56, 245–251. doi: 10.1016/j.vph.2012.02.006

Lukashev, D., and Sitkovsky, M. (2008). Preferential expression of the novel alternative isoform I.3 of hypoxia-inducible factor 1alpha in activated human T lymphocytes. *Hum. Immunol.* 69, 421–425. doi: 10.1016/j.humimm.2008.05.004

Machlus, K. R., and Italiano, J. J. E. (2013). The incredible journey: from megakaryocyte development to platelet formation. *J. Cell Biol.* 201, 785–796. doi: 10.1083/jcb.201304054

Magnani, M., Crinelli, R., Antonelli, A., Casabianca, A., and Serafini, G. (1994). The soluble but not mitochondrially bound hexokinase is a substrate for the ATP- and ubiquitin-dependent proteolytic system. *Biochim. Biophys. Acta* 1206, 180–190.

Metinko, A. P., Kunkel, S. L., Standiford, T. J., and Strieter, R. M. (1992). Anoxia-Hyperoxia induces monocyte-derived Interleukin-8. *J. Clin. Invest.* 90, 791–798.

Mizukami, Y., Jo, W. S., Duerr, E. M., Gala, M., Li, J., Zhang, X., et al. (2005). Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. *Nat. Med.* 11, 992–997. doi: 10.1038/nm1294

Mizukami, Y., Kohgo, Y., and Chung, D. C. (2007). Hypoxia inducible Factor-1 independent pathways in tumor angiogenesis. *Clin. Cancer Res.* 13, 5670–5674. doi: 10.1158/1078-0432.CCR-07-0111

Monsef, M., Soller, M., Panagopoulos, I., and Abrahamsson, P. A. (2010). HIF1α isoforms in benign and malignant prostate tissue and their correlation to neuroendocrine differentiation. *BMC Cancer* 10:385. doi: 10.1186/1471-2407-10-385

Morrison, S. J., and Scadden, D. T. (2014). The bone marrow niche for haematopoietic stem cells. *Nature* 505, 327–334. doi: 10.1038/nature12984

Munoz-Sànchez, J., and Chanez-Càrdenas, M. (2019). The use of cobalt chloride as a chemical hypoxia model. *J. Appl. Toxicol.* 39, 556–570. doi: 10.1002/jat.3749

Murdoch, C., Muthana, M., and Lewis, C. E. (2005). Hypoxia regulates macrophage functions in inflammation. *J. Immunol.* 175, 6257–6263. doi: 10.4049/jimmunol.175.10.6257

Palazon, A., Goldrath, A. W., Nizet, V., and Johnson, R. S. (2014). HIF transcription factors, inflammation, and immunity. *Immunity* 41, 518–528. doi: 10.1016/j.immuni.2014.09.008

Palma, L., Sfara, C., Antonelli, A., and Magnani, M. (2011). Pharmacology, dexamethasone restrains ongoing expression of interleukin-23p19 in peripheral blood-derived human macrophages. *BMC Pharmacol.* 11:8. doi: 10.1186/1471-2210-11-8

Park, E. C., Ghose, P., Shao, Z., Ye, Q., Kang, L., Xu, X. Z. S., et al. (2012). Hypoxia regulates glutamate receptor trafficking through an HIF-independent mechanism. *EMBO J.* 31, 1379–1393. doi: 10.1038/emboj.2011.499

Parmar, K., Mauch, P., Vergilio, J. A., Sackstein, R., and Down, J. D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *PNAS* 104, 5431–5436. doi: 10.1073/pnas.0701152104

Poulos, M. G., Guo, P., Kofler, N. M., Pinho, S., Gutkin, M. C., Tikhonova, A., et al. (2013). Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Rep.* 4, 1022–1034. doi: 10.1016/j.celrep.2013.07.048

Poussin, C., Gallitz, I., Schlage, W. K., Steffen, Y., Stolle, K., Lebrun, S., et al. (2014). Mechanism of an indirect effect of aqueous cigarette smoke extract on the adhesion of monocytic cells to endothelial cells in an in vitro assay revealed by transcriptomics analysis. *Toxicol. In Vitro* 28, 896–908. doi: 10.1016/j.tiv.2014.03.005

Rana, N. K., Singh, P., and Koch, B. (2019). $CoCl_2$ simulated hypoxia induce cell proliferation and alter the expression pattern of hypoxia associated genes involved in angiogenesis and apoptosis. *Biol. Res.* 52, 12–24. doi: 10.1186/s40659-019-0221-z

Richter, R., Forssmann, W., and Henschler, R. (2017). Current developments in mobilization of hematopoietic stem and progenitor cells and their interaction with niches in bone marrow. *Transfus. Med. Hemother.* 44, 151–164. doi: 10.1159/000477262

Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A. S., Nizet, V., et al. (2008). NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* 453, 807–811. doi: 10.1038/nature06905

Rydberg, E. K., Salomonsson, L., Hulten, L. M., Noren, K., Bondjers, G., Wiklund, O., et al. (2003). Hypoxia increases 25-hydroxycholesterol-induced interleukin-8 protein secretion in human macrophages. *Atherosclerosis* 170, 245–252. doi: 10.1016/s0021-9150(03)00302-2

Sanhueza, C., Bennett, J. C., Valenzuela-Valderrama, M., Contreras, P., Lobos-Gonzalez, L., Campos, A., et al. (2020). Caveolin-1-mediated tumor suppression is linked to reduced HIF1α S-Nitrosylation and transcriptional activity in hypoxia. *Cancers* 12, 2349–2366. doi: 10.3390/cancers12092349

Scarpa, E. S., Tasini, F., Crinelli, R., Ceccarini, C., Magnani, M., and Bianchi, M. (2020). The ubiquitin gene expression pattern and sensitivity to UBB and UBC knockdown differentiate primary 23132/87 and metastatic MKN45 gastric cancer cells. *Int. J. Mol. Sci.* 21, 5435–5453. doi: 10.3390/ijms21155435

Schuettpelz, L. G., and Link, D. C. (2013). Regulation of hematopoietic stem cell activity by inflammation. *Front. Immunol.* 4:204. doi: 10.3389/fimmu.2013.00204

Shih, S. C., and Claffey, K. P. (1998). Hypoxia-mediated regulation of gene expression in mammalian cells. *Int. J. Exp. Pathol.* 79, 347–357.

Singha, B., Gatla, H. R., Manna, S., Chang, T. P., Sanacora, S., Poltoratsky, V., et al. (2014). Proteasome inhibition increases recruitment of IkB kinase B (IKKB), S536P-p65, and transcription factor EGR1 to interleukin-8 (IL-8) promoter, resulting in increased IL-8 production in ovarian cancer cells. *J. Biol. Chem.* 289, 2687–2700. doi: 10.1074/jbc.M113.502641

Spencer, J. A., Ferraro, F., Roussakis, E., Klein, A., Wu, J., Runnels, J. M., et al. (2014). Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* 508, 269–273. doi: 10.1038/nature13034

Tamma, R., and Ribatti, D. (2017). Bone niches, hematopoietic stem cells, and vessel formation. *Int. J. Mol. Sci.* 18, 151–163. doi: 10.3390/ijms18010151

Tamura, R., Tanaka, T., Akasaki, Y., Murayama, Y., Yoshida, K., and Sasaki, H. (2020). The role of vascular endothelial growth factor in the hypoxic and immunosuppressive tumor microenvironment: perspectives for therapeutic implications. *Med. Oncol.* 37, 2–15. doi: 10.1007/s12032-019-1329-2

Tanaka, T., Narazaki, M., and Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. *Cold Spring Harb. Perspect. Biol.* 6:a016295. doi: 10.1101/cshperspect.a016295

Tardòn, M. C., Marinari, E., Migliorini, D., Bes, V., Tankov, S., Charrier, E., et al. (2020). An experimentally defined hypoxia gene signature in glioblastoma and its modulation by metformin. *Biology* 9, 264–280. doi: 10.3390/biology9090264

Toffoli, S., Feron, O., Raes, M., and Michiels, C. (2007). Intermittent hypoxia changes HIF-1α phosphorylation pattern in endothelial cells: Unravelling of a new PKA-dependent regulation of HIF-1α. *Biochim. Biophys. Acta* 1773, 1558–1571. doi: 10.1016/j.bbamcr.2007.06.002

Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. U. S. A.* 92, 5510–5514.

Xue, H., Yuan, G., Guo, X., Liu, Q., Zhang, J., Gao, X., et al. (2016). A novel tumor-promoting mechanism of IL6 and the therapeutic efficacy of tocilizumab: hypoxia-induced IL6 is a potent autophagy initiator in glioblastoma via the p-STAT3-MIR155-3p-CREBRF pathway. *Autophagy* 12, 1129–1152. doi: 10.1080/15548627.2016.1178446

Yuan, Y., Hilliard, G., Ferguson, T., and Millhorn, D. E. (2003). Cobalt inhibits the interaction between hypoxia inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. *J. Biol. Chem.* 278, 15911–15916. doi: 10.1074/jbc.M300463200

Zhang, P., Zhang, C., Li, J., Han, J., Liu, X., and Yang, H. (2019). The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications. *Stem Cell Res Ther.* 10, 327–339. doi: 10.1186/s13287-019-1422-7

Ziegler-Heitbrock, H. W., Thiel, E., Futterer, A., Herzog, V., Wirtz, A., and Riethmuller, G. (1988). Establishment of a human cell line mono mac 6 with characteristics of mature monocytes. *Int. J. Cancer* 41, 456–461. doi: 10.1002/ijc.2910410324

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Differential Brain and Muscle Tissue Oxygenation Responses to Exercise in Tibetans Compared to Han Chinese

Jui-Lin Fan[1], Tian Yi Wu[2,3], Andrew T. Lovering[4], Liya Nan[3], Wang Liang Bang[3] and Bengt Kayser[5]*

[1] Department of Physiology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, [2] Research Center for High Altitude Medicine, Tibet University Medical College, Lhasa, China, [3] National Key Laboratory of High Altitude Medicine, Xining, China, [4] Department of Human Physiology, University of Oregon, Eugene, OR, United States, [5] Institute of Sport Sciences, University of Lausanne, Lausanne, Switzerland

The Tibetans' better aerobic exercise capacity at altitude remains ill-understood. We tested the hypothesis that Tibetans display better muscle and brain tissue oxygenation during exercise in hypoxia. Using near-infrared spectrometry (NIRS) to provide indices of tissue oxygenation, we measured oxy- and deoxy-hemoglobin ([O_2Hb] and [HHb], respectively) responses of the vastus lateralis muscle and the right prefrontal cortex in ten Han Chinese and ten Tibetans during incremental cycling to exhaustion in a pressure-regulated chamber at simulated sea-level (air at 1 atm: normobaric normoxia) and 5,000 m (air at 0.5 atm: hypobaric hypoxia). Hypoxia reduced aerobic capacity by ∼22% in both groups (d = 0.8, p < 0.001 vs. normoxia), while Tibetans consistently outperformed their Han Chinese counterpart by ∼32% in normoxia and hypoxia (d = 1.0, p = 0.008). We found cerebral [O_2Hb] was higher in Tibetans at normoxic maximal effort compared Han (p = 0.001), while muscle [O_2Hb] was not different (p = 0.240). Hypoxic exercise lowered muscle [O_2Hb] in Tibetans by a greater extent than in Han (interaction effect: p < 0.001 vs. normoxic exercise). Muscle [O_2Hb] was lower in Tibetans when compared to Han during hypoxic exercise (d = 0.9, p = 0.003), but not during normoxic exercise (d = 0.4, p = 0.240). Muscle [HHb] was not different between the two groups during normoxic and hypoxic exercise (p = 0.778). Compared to Han, our findings revealed a higher brain tissue oxygenation in Tibetans during maximal exercise in normoxia, but lower muscle tissue oxygenation during exercise in hypoxia. This would suggest that the Tibetans privileged oxygenation of the brain at the expense of that of the muscle.

Keywords: Tibetans, exercise performance, hypoxia, muscle tissue oxygenation, cerebral tissue oxygenation

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****Correspondence:***
Jui-Lin Fan
mickey.fan@auckland.ac.nz

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INTRODUCTION

Aerobic performance is an important determinant of one's ability to thrive at high altitude, yet the exact mechanisms limiting aerobic performance at high altitude remain poorly understood. Tibetans are renowned for their superior exercise performance at high altitude compared to their Western and Han Chinese counterparts (Wu et al., 2005; Marconi et al., 2006; Wu and Kayser, 2006;

Gilbert-Kawai et al., 2014; Kayser et al., 2019). While this remarkable performance has been largely attributed to better systemic oxygen (O_2) transport in Tibetans, it is unknown whether this translates into better muscle and/or brain tissue oxygenation during exercise.

Within the skeletal muscles, Tibetans and Sherpas display lower mitochondrial volume density and muscle fiber cross-sectional area, and higher muscle capillary density and myoglobin concentration compared to lowlanders (Kayser et al., 1991, 1996; Erzurum et al., 2007). Furthermore, Tibetans have been reported to display lower O_2 consumption for a given exercise workload compared to lowlanders, suggesting a better O_2 economy (Ge et al., 1994). But how these differences in muscle ultrastructure and O_2 economy relate to oxidative metabolism in the working muscle has not been investigated. By comparing muscle tissue oxygenation in Tibetans and their Han Chinese counterparts during exercise in normoxia and hypoxia, we aimed to gain further insight into the functional differences in skeletal muscle oxidative metabolism between these two groups.

Near-infrared spectroscopy (NIRS) provides a non-invasive method of assessing changes in muscle tissue oxygenation during exercise (Perrey and Ferrari, 2017). By determining the relative changes in oxy- and deoxy-hemoglobin concentrations ([O_2Hb] and [HHb], respectively), muscle NIRS signals provide unique insights into O_2 balance (and therefore oxidative metabolism) in the working muscle (Grassi and Quaresima, 2016; Perrey and Ferrari, 2017). In the brain, NIRS-determined capillary oxygenation is functionally related to the balance between O_2 saturation of arterial and venous blood, and has been shown to reflect cerebral capillary oxygenation-level-dependent changes (Rasmussen et al., 2007). Studies have reported an association between performance and NIRS-derived cerebral tissue deoxygenation in severe hypoxia (arterial oxygen saturation [SaO_2] < 75%) during repeated sprints (Smith and Billaut, 2010), incremental exercise (Subudhi et al., 2007; Peltonen et al., 2009), and static maximal or sub-maximal muscle contraction to exhaustion (Rasmussen et al., 2007; Rupp and Perrey, 2009; Vogiatzis et al., 2011; Millet et al., 2012).

It has been speculated that cerebral tissue oxygenation may play a pivotal role in the limitation of performance during exercise in severe hypoxia [(Kjaer et al., 1999; Amann et al., 2006, 2007; Rasmussen et al., 2010), see Fan and Kayser (2016) for review]. During incremental cycling at 3,658 m, Tibetans have been reported to exhibit higher internal carotid blood flow and cerebral O_2 delivery compared to Han Chinese (Huang et al., 1992). Whether these differences translate to higher cerebral tissue oxygenation, and thereby could partly account for the superior performance in Tibetans is unknown.

The purpose of this study was to compare the muscle and cerebral tissue oxygenation responses to exercise in a pressure-regulated chamber in normobaric normoxia (air at 1 atm, to simulate sea-level) and hypobaric hypoxia (air at 0.5 atm, equivalent to ∼5,000 m altitude) in Tibetans and Han Chinese living in Xining (2,260 m) of the Qinghai Province, People's Republic of China. We tested the hypothesis that when compared to Han Chinese, Tibetans would exhibit higher brain and muscle tissue oxygenation during exercise in hypoxia, but not during exercise in normoxia.

MATERIALS AND METHODS

Participants

Forty male participants were initially recruited into this study. Following saline-contrast echocardiography screening to exclude participants with a patent foramen ovale (see below), twenty male participants completed this study, consisting of ten Han Chinese and ten Tibetans (**Table 1**). The Tibetan group consisted of individuals of Tibetan descent who were born and raised at altitudes of >3,500 m and currently residing in Xining of the Qinghai Province, Peoples Republic of China, while the Han Chinese group were all second-generation permanent residents of Xining of the Qinghai Province, Peoples Republic of China. To minimize the confounding influence of training status and physical activity, we recruited recreationally active individuals.

All the participants were judged healthy by medical history, physical examination, resting electrocardiogram, echocardiogram and respiratory function tests and were not taking any medication. The participants were informed of the experimental procedures and potential risks involved in the study before their written consent was obtained. The study was approved by the University of Oregon Institutional Review Board and the Qinghai High Altitude Medical Science Institutional Committee on Human Research and complied with the *Declaration of Helsinki*.

Saline-Contrast Echocardiography Screening

All the participants underwent saline-contrast echocardiography with and without performing a Valsalva maneuver to screen for the presence of patent foramen ovale (PFO). The foramen ovale is an interatrial communication which allows blood flow to bypass the pulmonary circulation during fetal life but normally closes after birth. Importantly, the presence of a PFO in adults has been shown to elevate resting alveolar-arterial O_2 gradient (i.e., reduced pulmonary gas exchange efficiency) and lower SaO_2 during maximal exercise in normoxia (Lovering et al., 2011). Likewise, the presence of a PFO is believed to exacerbate arterial

TABLE 1 | Participant characteristics of Han Chinese and Tibetans (values concern acute normobaric normoxic condition).

	Han Chinese	Tibetan	*p*-value
n	**10**	**10**	
Age(years)	23.3 ± 1.6	21.2 ± 3.1	0.078
Height(cm)	173.9 ± 6.4	171.0 ± 6.1	0.531
Body mass (kg)	62.2 ± 7.7	64.4 ± 7.7	0.314
BMI (kg m^{-2})	20.5 ± 1.9	22.0 ± 1.9	0.108
$\dot{V}O_2$ peak (ml.min^{-1}.kg)	35.9 ± 4.1	38.9 ± 10.2	0.408
[Hb] (g.L^{-1})	16.5 ± 1.7	16.0 ± 3.2	0.724
Hct (%)	55.7 ± 2.1	56.2 ± 3.5	0.658

BMI, body mass index; $\dot{V}O_2$ peak, O_2 uptake at 1 atm; [Hb], Hemoglobin concentration; Hct, hematocrit concentration.

hypoxemia in severe chronic obstructive pulmonary disease patients at rest (Allemann et al., 2006; Brenner et al., 2015; Elliott et al., 2015). Therefore, to avoid the possible confounding influence of a PFO, those who were determined to have a PFO were excluded from the study. Similarly as previously found in Sherpas living at high altitude (Foster et al., 2014), the prevalence of PFO was 50% in our cohort of Tibetans.

Experimental Design

The participants visited the laboratory on three occasions. After a full familiarization with the experimental procedures outlined below (visit one), the participants underwent two experimental exercise sessions (visits two and three) in a pressure-regulated chamber (3 m × 8 m × 3 m hyper- and hypobaric pressure chamber, Shanghai Far East Petroleum Machinery Co., China): (i) normobaric normoxia (simulated sea-level by increasing the chamber pressure to 1 atm), and (ii) hypobaric hypoxia (simulated 5,000 m by decreasing the chamber pressure to 0.5 atm), in a randomized, single-blinded, balanced fashion. Due to technical limitations with the chamber, there was a mild increase in fraction of inspired O_2 (FIO_2) from 0.209 to 0.22–0.24 resulting in a mild increase in inspired O_2 (see methodological considerations).

For the normobaric normoxia and hypobaric hypoxia sessions, the experimental protocol was comprised of: (i) 20 min instrumentation; (ii) 4 min hyperoxic exposure, breathing 100% O_2 through a face mask, followed by a 3-min washout period to ensure all the variables returned to pre-O_2 breathing values; (iii) 4 min resting baseline; (iv) step-incremental cycling until exhaustion; (v) 5 min recovery; and (vi) ramp-incremental cycling until exhaustion. All of the experimental sessions were conducted at the National Key Laboratory of High Altitude Medicine in the city of Xining of the Qinghai Province, People's Republic of China. Before each experimental session, all participants were asked to abstain from caffeine for 12 h, and alcohol for 24 h.

Exercise Tests

Step-Incremental Cycling Until Exhaustion

Seated on a reclining ergometer tilted into a left lateral position (Ergoselect 1000, Ergoline GmbH, Bitz, Germany), the participants were instructed to begin cycling at 70 watts, at a pedaling rate of 70 rpm. The work rate was increased by 30 watts every 3 min thereafter until the participant reached voluntary exhaustion.

Ramp Incremental Cycling to Exhaustion

Following a 5-min recovery period whilst positioned on the ergometer, the participants were instructed to begin cycling at 0 watts, at a pedaling rate of 70 rpm. The work rate was increased by 0.5 watts every second (i.e., equivalent of 30 watts.min^{-1}) thereafter until the participant reached voluntary exhaustion.

Measurements

Respiratory Variables

Throughout the experimental protocol, the participants wore a facemask attached to a spirometer (TripleV-volume sensor, CareFusion, San Diego, CA, United States), from which expired gases and breath-by-breath respiratory flow was monitored using a metabolic cart (Jaeger, CareFusion, San Diego, CA, United States). Ventilation ($\dot{V}E$), O_2 uptake ($\dot{V}O_2$), expired CO_2 ($\dot{V}CO_2$), and respiratory exchange ratio (RER) were then calculated by the metabolic cart and expressed in either L.min^{-1} BTPS (for $\dot{V}E$) or mL.min^{-1} STPD ($\dot{V}O_2$ and $\dot{V}CO_2$).

Cardiovascular and Cerebrovascular Variables

Continuous beat-to-beat blood pressure was monitored using finger plethysmography (Finometer MIDI, Finapres Medical Systems, Amsterdam, Netherlands), from which mean blood pressure (BP) was derived from the timed-average of the BP waveform. Peripheral O_2 saturation (SpO_2) was measured from the right side of the forehead using pulse oximetry (N-200, Nellcor Inc., Hayward, CA, United States). A three-lead electrocardiogram was used to determine heart rate (HR). Stroke volume (SV) was estimated using transthoracic electrical bioimpedance cardiography (PhysioFlow®, Manatec PF07 Enduro, Paris, France). Cardiac output (CO) was subsequently calculated by multiplying SV with HR.

Bilateral middle cerebral artery blood velocities (MCAv, an index of cerebral blood flow) were measured in the middle cerebral artery using a 2-MHz pulsed Doppler ultrasound system (ST3, Spencer Technology, Seattle, OR, United States). The Doppler ultrasound probes were positioned over the temporal windows and held in place with an adjustable plastic headband. The MCAv signals were acquired at depths ranging from 43 to 54 mm. Signal quality was optimized, and an M-mode screen shot was recorded to facilitate subsequent probe placements. In our hands, day-to-day reproducibility of MCAv has a coefficient of variation of <10%. The bilateral MCAv were averaged using the following equation to represent global cerebral blood flow (CBF) during rest and exercise:

$$\text{mean MCAv} = \left[\frac{\text{left MCAv} + \text{right MCAv}}{2}\right] \quad (1)$$

Muscle and Cerebral Tissue Oxygenation

Muscle tissue oxygenation in the left vastus lateralis muscle (∼15 cm proximal and 5 cm lateral to the superior border of the patella) was measured by monitoring changes in [O_2Hb] and [HHb] concentrations obtained using spatially resolved, continuous wave near-infrared spectroscopy (NIRS, Oxymon MKIII, Artinis, Zetten, Netherlands). For the muscle tissue oxygenation, a source-detector spacing of 3.8 cm and a differential pathlength factor of 4.0 were used (Duncan et al., 1995). Cerebral tissue oxygenation in the left prefrontal lobe was assessed with an additional NIRS channel of the same instrument. Both muscle and cerebral NIRS channels were zeroed at rest and expressed as absolute values. For the cerebral tissue oxygenation, source-detector spacing was set at 4.1 cm and data obtained from the optodes were used to calculate changes in [O_2Hb] and [HHb] with a differential pathlength factor (DPF) calculated using the formula: DPF = 4.99 + 0.067 × age$^{0.814}$ (Duncan et al., 1995). Total Hb ([totHb]) was calculated using the equation:

$$[\text{totHb}] = [O_2\text{Hb}] + [\text{HHb}] \quad (2)$$

The cerebral and muscle [O_2Hb], [HHb], and [totHb] are expressed as absolute values (μMol).

Arterial Blood Gases

Arterial blood gas samples were obtained from a 22-gauge arterial catheter placed into a radial artery; blood samples (2 mL) were taken over approximately five cardiac cycle periods into a pre-heparinized syringe. Following standardized calibration, all blood samples were analyzed using an arterial blood-gas analyzing system (ABL 77 Sci, Radiometer, Copenhagen, Denmark) for pH, partial pressure of arterial O_2 (PaO_2) and CO_2 ($PaCO_2$), arterial O_2 saturation (SaO_2), hemoglobin concentration ([Hb]) and hematocrit (Hct). Arterial O_2 content (CaO_2) was subsequently calculated using the equation:

$$CaO_2 = [Hb] \times 1.36 \times \left[\frac{SaO_2}{100}\right] + PaO_2 \times 0.003 \quad (3)$$

Aural temperature from the right ear was recorded as a surrogate of core body temperature. The blood gas values were temperature corrected (Kelman and Nunn, 1966; Severinghaus, 1966).

Energy Economy

Energy expended (EE) was calculated using the equation (Moseley and Jeukendrup, 2001):

$$EE = [(\dot{V}O_2 \times 3.869) + (\dot{V}CO_2 \times 1.195) \times (4.186/60) \times 1000]$$

where, EE is energy expended in $J.s^{-1}$, $\dot{V}O_2$ is O_2 uptake in $L.min^{-1}$ and $\dot{V}CO_2$ is the CO_2 expired in $L.min^{-1}$.

Gross efficiency (GE) during steady-state exercise was calculated using the equation (Moseley and Jeukendrup, 2001):

$$GE = \text{Work rate}/EE \times 100 \quad (4)$$

Where GE is gross efficiency in percentage, work rate is in watts, and EE is energy expended in $J.s^{-1}$.

Exercise economy (EC) was calculated as power output divided by O_2 uptake and expressed as $kJ.L^{-1}$.

Except for $\dot{V}E$, $\dot{V}O_2$, $\dot{V}CO_2$ and RER which were recorded on the metabolic cart, all analog data were sampled and recorded at 200 Hz on a computer for off-line analysis (Powerlab 16/30, ADInstruments, Dunedin, New Zealand).

Statistical Analysis

Unpaired *t* test with Welch's correction was used to compare the participants' characteristics between Han Chinese and Tibetan (Prism 8, GraphPad Software, San Diego, CA, United States). Analyses of resting parameters were performed on averaged data from the last 2 min of the baseline period, and from the last 1 min of the hyperoxic exposure and recovery period. During exercise, the mean values during the last 30 s of workload (i.e., 70 watts, 100 watts, 130 watts, etc.), and the mean value of the last 30 s of the ramp incremental exercise for maximal exercise effort (MAX) were extracted for analysis.

The main effects of experimental condition (normoxia vs. hypoxia) and group (Han Chinese vs. Tibetans) on arterial blood gases, cardiorespiratory variables, cerebral haemodynamics and muscle tissue oxygenation during 100% O_2 breathing, at rest, during steady-state exercise (70 watts, 100 watts, 130 watts, and 160 watts), recovery and maximal exercise effort were assessed using mixed linear model analysis (SPSS Statistics version 23, IBM Corporation, Armonk, NY, United States). For significant effects and interaction between hypoxia effect and group effect, *post hoc* tests were performed using Sidak's adjustment for multiple comparisons (α-level of 0.05). In addition to *p*-values, Cohen's *d* values (effect size) are reported for altitude and group effects. Cohen's *d* value was calculated using the formula (Cohen, 1977):

$$d = \left[\frac{M_1 - M_2}{\sigma_{pooled}}\right] \quad (5)$$

where, M_1 and M_2 are means of group 1 and 2; σ_{pooled} is the standard deviation of the pooled data. The effect sizes were classified as (Sullivan and Feinn, 2012): negligible ($d < 0.2$); small ($d \geq 0.2$); medium ($d \geq 0.5$); large ($d \geq 0.8$); and very large ($d \geq 1.3$). Data are reported as mean ± SD in text, tables and figures.

RESULTS

Hyperoxia

In hypoxic conditions, 100% O_2 breathing increased muscle [O_2Hb] by ∼2.9 μM in both groups ($F = 7.8$, $d = 0.8$, $p = 0.012$), while neither muscle [HHb] ($F = 1.3$, $p = 0.264$) nor [totHb] were affected ($F = 1.2$, $p = 0.275$). There was a tendency for muscle [HHb] to be lower by ∼2.1 μM in Tibetans during 100% O_2 breathing ($F = 3.5$, $d = 0.4$, $p = 0.071$ vs. Han Chinese, **Figure 4**). In both groups, 100% O_2 breathing decreased MCAv by ∼7.3 $cm.s^{-1}$ in hypoxia compared to normoxia ($F = 5.4$, $d = 0.5$, $p = 0.032$), while no significant group effects were observed ($p > 0.05$).

Performance

Hypoxia reduced aerobic capacity by ∼22% in both Han Chinese and Tibetans compared to their normoxic values ($F = 31.5$, $d = 0.8$, $p < 0.001$, **Figure 1**). Irrespective of the experimental condition, Tibetans performed significantly better than their Han Chinese counterparts by ∼32% during incremental exercise to exhaustion ($F = 8.8$, $d = 1.0$, $p = 0.008$).

Resting, Steady-State Exercise and Recovery

Arterial Blood Gases

At rest, lowering barometric pressure reduced PaO_2 by ∼85.6 mmHg ($F = 1778.1$), $PaCO_2$ by ∼6.3 mmHg ($F = 112.7$), SaO_2 by ∼16.8% ($F = 132.8$) and CaO_2 by ∼3.0 mL $O_2.dl^{-1}$ ($F = 10.51$), and elevated resting pH by ∼0.05 ($F = 60.5$) in both

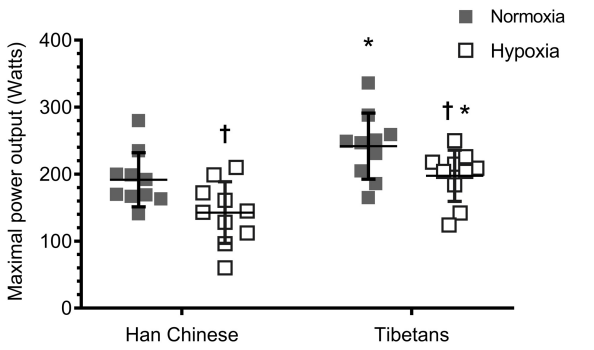

FIGURE 1 | Aerobic capacity during ramp incremental cycling to exhaustion in Han Chinese and Tibetans. *different from Han Chinese, $p < 0.05$; †different from normoxia. Data expressed mean ± SD.

Han Chinese and Tibetans ($d > 1.0$ and $p < 0.01$ vs. normoxia for all, **Table 2**).

On average, hypoxia reduced PaO_2 by ∼76.3 mmHg throughout step-incremental cycling ($F = 8682.7$, $d = 2.0$), $PaCO_2$ by ∼13.7 mmHg ($F = 554.4$, $d = 1.5$), SaO_2 by ∼17.5% ($F = 2240.9$, $d = 1.0$), CaO_2 by ∼3.2 mL O_2.dl^{-1} ($F = 81.8$, $d = 1.1$) in both Han Chinese and Tibetans, while it elevated [Hb] by ∼0.8 g.L^{-1} ($F = 9.1$, $d = 0.5$) and pH by ∼0.09 ($F = 92.9$, $d = 1.5$, $p < 0.001$ for all, **Table 3**). There was no other difference between Han Chinese and Tibetans in any of the arterial blood gas parameters during exercise in both hypoxic and normoxic conditions ($p > 0.05$, **Table 3**).

During maximal effort, hypoxia lowered PaO_2 by ∼72.8 mmHg ($F = 1569.0$, $d = 1.9$), $PaCO_2$ by ∼16.7 mmHg ($F = 259.2$, $d = 1.7$), SaO_2 by ∼16.6% ($F = 66.1$, $d = 1.9$), CaO_2 by ∼2.9 mL O_2.dl^{-1} in both groups ($F = 12.3$, $d = 1.0$), while pH was elevated by ∼0.10 compared to normoxia ($F = 66.1$, $d = 1.7$, $p < 0.001$ for all, **Table 3**). There were no group effects on arterial blood gas parameters ($p > 0.05$, **Table 3**).

Cardiorespiratory

In both Han Chinese and Tibetans, exposure to hypoxia elevated resting HR by ∼19.6 b.min^{-1} ($F = 58.5$, $d = 1.2$), CO by ∼1.0 L.min^{-1} ($F = 6.1$, $d = 0.6$) and lowered SpO_2 by ∼17.7% ($F = 152.8$, $d = 1.8$) compared to normoxia ($p < 0.05$ vs.

TABLE 2 | Resting cardiorespiratory, cerebral haemodynamics and muscle tissue oxygenation in Han Chinese and Tibetans in normobaric normoxia and hypobaric hypoxia.

	Han Chinese		Tibetan		Main effects (*p*-values)		
n	**10**		**10**				
Arterial blood gases	**Normoxia**	**Hypoxia**	**Normoxia**	**Hypoxia**	**Condition**	**Group**	**Interaction**
PaO_2 (mmHg)	128.8 ± 7.5	45.6 ± 4.9†	135.1 ± 8.9	47.2 ± 7.3†	**0.000**	0.158	0.263
$PaCO_2$ (mmHg)	40.2 ± 5.2	33.0 ± 3.8†	37.7 ± 4.2	32.4 ± 4.1†	**0.000**	0.417	0.126
pH	7.35 ± 0.02	7.41 ± 0.03†	7.37 ± 0.03	7.41 ± 0.03†	**0.000**	0.319	0.091
SaO_2 (%)	98.8 ± 0.2	81.2 ± 4.0†	99.0 ± 0.1	83.0 ± 8.4†	**0.001**	0.503	0.613
CaO_2 (mL O_2.dl^{-1})	22.2 ± 2.3	19.3 ± 2.0†	21.7 ± 4.2	18.6 ± 2.6†	**0.005**	0.518	0.879
Cardiorespiratory							
BP (mmHg)	105.7 ± 8.8	102.3 ± 8.2	103.8 ± 10.3	99.3 ± 8.8	0.177	0.396	0.856
HR (b.min^{-1})	74 ± 11	90 ± 13†	72 ± 12	95 ± 19†	**0.001**	0.729	0.229
CO (L.min^{-1})	7.3 ± 1.3	7.1 ± 1.3†	8.1 ± 1.9	8.3 ± 1.7†	**0.024**	0.959	0.708
$\dot{V}E$ (L.min^{-1})	17.3 ± 2.9	14.7 ± 1.5†	17.9 ± 2.6	16.1 ± 2.2†	**0.005**	0.276	0.550
$\dot{V}O_2$ (ml.min^{-1})	365 ± 40	376 ± 56	347 ± 45	383 ± 57	0.167	0.658	0.328
$\dot{V}CO_2$ (ml.min^{-1})	340 ± 46	323 ± 43	304 ± 41	337 ± 53	0.579	0.481	0.099
RER	0.92 ± 0.05	0.86 ± 0.07†	0.88 ± 0.04	0.88 ± 0.05	0.060	0.517	**0.047**
SpO_2 (%)	98.8 ± 1.1	78.4 ± 8.0†	99.0 ± 0.7	83.9 ± 4.3†	**0.001**	0.054	0.074
Cerebral haemodynamics							
MCAv (cm.s^{-1})	63.1 ± 16.9	65.2 ± 13.6	54.4 ± 9.6	58.3 ± 8.6	0.302	0.134	0.808
Cerebral [O_2Hb] (μmol)	0.9 ± 3.0	0.3 ± 2.4	1.4 ± 2.6	1.7 ± 3.8	0.321	0.888	0.649
Cerebral [HHb] (μmol)	−0.2 ± 1.5	−0.5 ± 2.2†	2.4 ± 3.9	2.2 ± 3.0†	**0.005**	0.793	0.956
Cerebral [totHb] (μmol)	0.7 ± 3.7	−0.1 ± 4.1†	3.8 ± 4.7	3.9 ± 5.5†	**0.017**	0.800	0.728
Muscle oxygenation							
Muscle [O_2Hb] (μmol)	1.2 ± 2.3	3.0 ± 2.7	3.4 ± 4.5	1.9 ± 4.7	0.651	0.906	0.174
Muscle [HHb] (μmol)	−0.6 ± 3.6	−0.5 ± 2.5†	1.9 ± 3.3	1.8 ± 2.5†	**0.016**	0.992	0.876
Muscle [totHb] (μmol)	0.6 ± 3.4	2.5 ± 3.2	5.3 ± 7.2	3.7 ± 5.1	0.070	0.924	0.265

PaO_2, partial pressure of arterial O_2; $PaCO_2$, partial pressure of arterial CO_2; SaO_2, arterial O_2 saturation; CaO_2, arterial O_2 content; BP, blood pressure; HR, heart rate; CO, cardiac output; $\dot{V}E$, pulmonary ventilation; $\dot{V}O_2$, O_2 uptake; $\dot{V}CO_2$, expired CO_2; RER, respiratory exchange ratio; SpO_2, peripheral O_2 saturation; MCAv, middle cerebral artery velocity; [O_2Hb], oxy-hemoglobin concentration; [HHb], deoxy-hemoglobin concentration; [totHb], total hemoglobin concentration. Values are mean ± SD. †different from normoxia ($p < 0.05$). Bold text indicate significant main effect ($p < 0.05$).

TABLE 3 | Arterial blood gases in Han Chinese and Tibetans during exercise at simulated sea-level and 5,000 m.

		Han Chinese		Tibetan		Main effects (*p*-values)		
n		10		10				
		Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**	**Condition**	**Group**	**Interaction**
PaO_2 (mmHg)	70 W	128.8 ± 7.5	45.4 ± 2.9†	126.9 ± 10.1	46.3 ± 4.6†	**0.001**	0.474	0.131
	100 W	121.9 ± 9.3	47.3 ± 5.2†	124.9 ± 7.6	46.9 ± 3.8†			
	130 W	120.8 ± 8.0	–	124.2 ± 10.2	48.0 ± 4.5†			
	160 W	–	–	122.0 ± 8.9	–			
	Max	124.3 ± 8.4	50.8 ± 4.6†	122.9 ± 9.5	49.5 ± 3.5†	**0.001**	0.658	0.962
$PaCO_2$ (mmHg)	70 W	46.2 ± 7.1	33.2 ± 4.6†	42.7 ± 4.7	31.8 ± 4.0†	**0.001**	0.575	0.160
	100 W	45.5 ± 7.7	28.5 ± 5.3†	43.4 ± 4.9	29.5 ± 4.4†			
	130 W	47.0 ± 5.6	–	42.2 ± 5.9	25.5 ± 4.1†			
	160 W	–	–	42.5 ± 6.2	–			
	Max	39.1 ± 5.5	22.9 ± 3.3†	41.8 ± 5.8	24.1 ± 4.3†	**0.001**	0.310	0.592
pH (a.u.)	70 W	7.30 ± 0.04	7.36 ± 0.03†	7.32 ± 0.03	7.38 ± 0.04†	**0.001**	0.303	0.623
	100 W	7.26 ± 0.05	7.33 ± 0.04†	7.29 ± 0.03	7.35 ± 0.02†			
	130 W	7.22 ± 0.07	–	7.26 ± 0.03	7.31 ± 0.03†			
	160 W	–	–	7.22 ± 0.04	–			
	Max	7.19 ± 0.06	7.29 ± 0.03†	7.19 ± 0.05	7.28 ± 0.04†	**0.001**	0.988	0.954
SaO_2 (%)	70 W	98.2 ± 0.4	79.7 ± 2.5†	98.6 ± 0.3	81.9 ± 4.5†	**0.001**	0.286	0.121
	100 W	98.0 ± 0.6	80.0 ± 4.6†	98.4 ± 0.2	81.4 ± 2.8†			
	130 W	97.6 ± 0.7	–	98.2 ± 0.3	81.1 ± 3.7†			
	160 W	–	–	97.9 ± 0.3	–			
	Max	97.6 ± 0.8	81.2 ± 4.7†	97.6 ± 0.9	80.5 ± 3.2†	**0.001**	0.804	0.752
[Hb] ($g.L^{-1}$)	70 W	16.3 ± 2.5	17.6 ± 1.0	16.2 ± 2.5	16.6 ± 1.2	**0.003**	0.257	0.190
	100 W	17.0 ± 2.1	18.3 ± 2.0	16.1 ± 2.3	16.8 ± 1.2			
	130 W	17.1 ± 1.8	–	16.9 ± 1.1	17.7 ± 0.8			
	160 W	–	–	17.3 ± 1.4	–			
	Max	17.8 ± 2.5	19.3 ± 1.7	17.2 ± 1.8	18.0 ± 1.1	0.057	0.189	0.543
CaO_2 (ml $O_2.dl^{-1}$)	70 W	21.9 ± 3.3	19.0 ± 1.4†	21.8 ± 3.2	18.3 ± 2.0†	**0.001**	0.443	0.488
	100 W	22.7 ± 2.7	19.9 ± 3.1†	21.6 ± 3.0	18.5 ± 1.5†			
	130 W	22.8 ± 2.3	–	22.6 ± 1.4	19.3 ± 1.2†			
	160 W	–	–	23.0 ± 1.8	–			
	Max	23.6 ± 3.2	21.2 ± 2.5†	22.9 ± 2.2	19.6 ± 1.2†	**0.001**	0.163	0.623

PaO_2, partial pressure of arterial O_2; $PaCO_2$, partial pressure of arterial CO_2; SaO_2, arterial O_2 saturation; [Hb], hemoglobin concentration; CaO_2, arterial O_2 content. Values are mean ± SD. †different from normoxia ($p < 0.05$). Bold text indicate significant main effect ($p < 0.05$).

normoxia for all, **Table 2**). There was a trend for Tibetans to display higher resting SpO_2 compared to the Han Chinese group ($F = 3.9$, $p = 0.054$), particularly in hypoxia (interaction: $F = 3.4$, $p = 0.074$, **Table 2**). *Post hoc* pairwise analysis showed resting SpO_2 was higher in Tibetans by ∼5.5% in hypoxia compared to Han ($d = 0.8$, $p = 0.011$), but was not different in normoxia ($p = 0.917$). Hypoxia selectively lowered resting RER in Han Chinese (interaction: $F = 4.6$, $p = 0.047$). As a result, hypoxia lowered resting RER in Han Chinese by ∼0.054 ($d = 0.8$, $p = 0.009$ vs. normoxia), while no change was observed in Tibetans ($d < 0.1$, $p = 0.929$).

For a given workload, hypoxia elevated HR by ∼10.2 $b.min^{-1}$ ($F = 5.8$, $d = 0.5$, $p = 0.017$) and lowered SpO_2 by ∼20.1% ($F = 965.9$, $d = 1.5$, $p < 0.001$), $\dot{V}O_2$ by ∼283 $ml.min^{-1}$ ($F = 11.9$, $d = 0.6$, $p = 0.001$) and $\dot{V}CO_2$ by ∼251 $ml.min^{-1}$ ($F = 80.1$, $d = 0.5$, $p < 0.001$), but had no effects on CO or BP ($F \leq 0.5$, $p > 0.05$, **Figures 2**, **3**). We observed no significant group effects on HR, SpO_2, $\dot{V}O_2$ or CO during step-incremental cycling ($p > 0.05$). There was a group difference on RER during step-incremental cycling ($F = 9.4$, $p = 0.002$), which was mediated by a non-significant group difference in $\dot{V}CO_2$ ($F = 6.0$, $d = 0.2$, $p = 0.054$). In both normoxia and hypoxia, RER was lower in Tibetans by ∼0.097 ($d = 1.1$) throughout the step-incremental cycling compared to Han Chinese.

During recovery, hypoxia lowered SpO_2 by ∼11.9% ($F = 161.2$, $d = 1.8$, $p < 0.001$) compared to normoxia, but had no effects on HR ($F = 0.0$, $p = 0.872$) or BP ($F = 1.3$, $p = 0.269$, **Figure 2**). There were no group differences in cardiorespiratory parameters during recovery ($p > 0.05$).

During maximal effort, hypoxia lowered SpO_2 by ∼15.6% ($F = 62.5$, $d = 1.5$, $p < 0.001$), $\dot{V}O_2$ by ∼462 $ml.min^{-1}$ ($F = 18.2$, $d = 0.9$, $p = 0.001$)), $\dot{V}CO_2$ by ∼462 $ml.min^{-1}$ ($F = 15.9$, $d = 0.8$, $p < 0.001$) and CO by ∼1.7 $L.min^{-1}$ ($F = 5.5$, $d = 0.6$, $p = 0.031$), but had no effect on HR or BP ($p > 0.05$, **Figure 2**, **3**). Irrespective

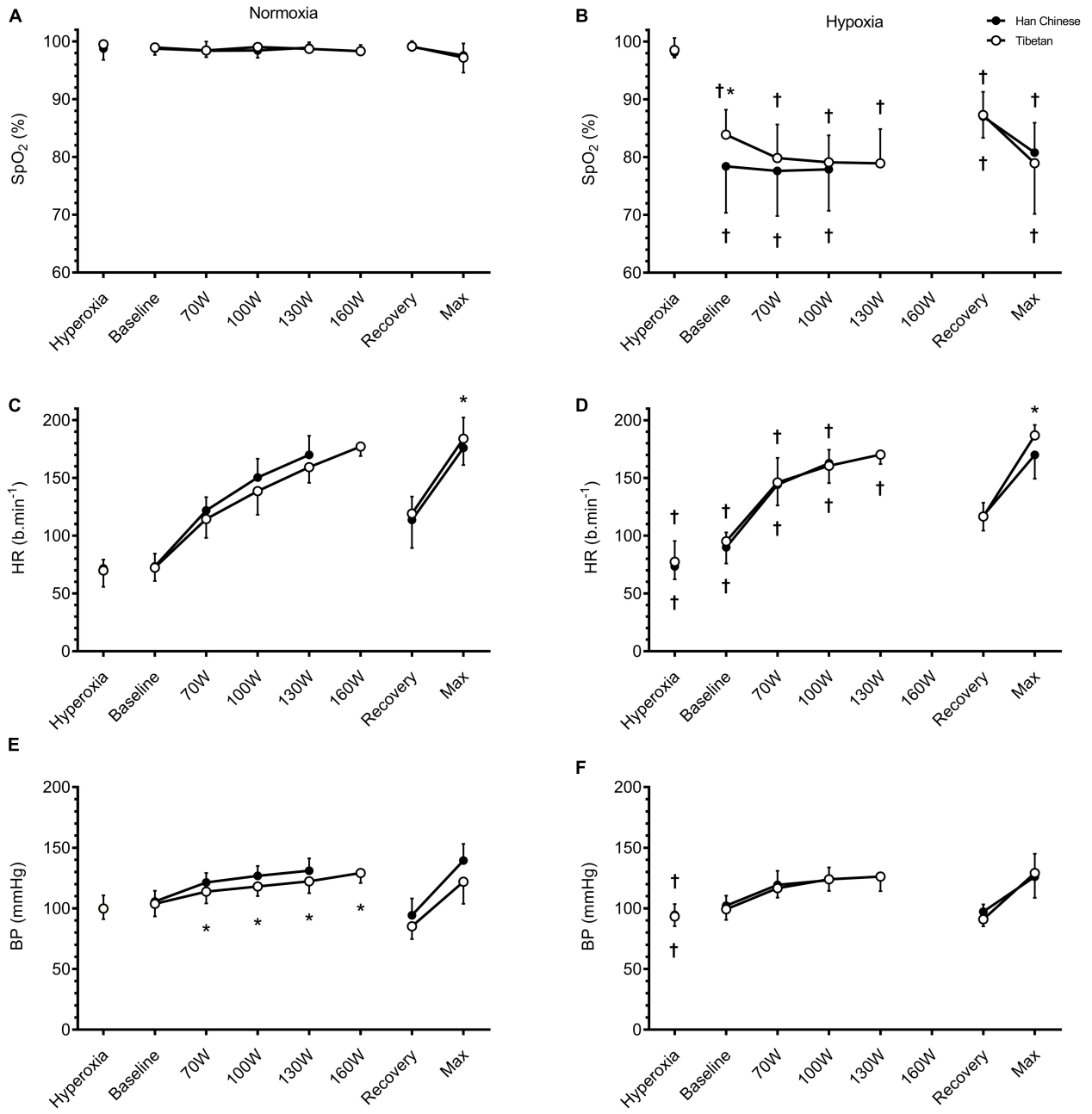

FIGURE 2 | Cardiovascular responses during hyperoxia, baseline, step-incremental cycling, recovery and ramp-incremental cycling to exhaustion (Max) in Han Chinese and Tibetans. Left panel, normoxia: Right panel, hypoxia (equivalent of ∼5,000 m altitude). SpO_2, peripheral O_2 saturation; HR, heart rate; BP, blood pressure. *different from Han Chinese, $p < 0.05$; †different from normoxia, $p < 0.05$. Data expressed mean ± SD.

of the experimental conditions, Tibetans had higher HR (by ∼17.5 b.min^{-1}, $F = 6.3$, $p = 0.017$, **Figure 2**) and CO at maximal exercise compared to Han Chinese (by ∼3.2 L.min^{-1}, $F = 13.6$, $d = 1.1$, $p = 0.002$).

Energy Economy

No condition or group effects were observed in EE, GE or EC during step-incremental cycling ($p < 0.05$, **Table 4**). Hypoxia lowered EE at maximal effort by ∼370 J.s^{-1} ($F = 10.5$, $d = 1.9$, $p = 0.005$). Irrespective of the condition, GE was higher by ∼5%, in Tibetans at maximal effort compared to Han Chinese ($F = 10.7$, $d = 1.1$, $p = 0.004$), while EC was higher by ∼0.18 kJ.L^{-1} ($F = 5.3$, $d = 0.9$, $p = 0.034$, **Table 4**).

Muscle Tissue Oxygenation

In both groups, hypobaric hypoxia elevated resting muscle [HHb] by ∼2.4 μM ($F = 6.4$, $d = 0.8$, $p = 0.016$), but had no effects on muscle [totHb] ($F = 3.4$, $p = 0.070$) or [O_2Hb] ($F = 0.2$, $p = 0.651$).

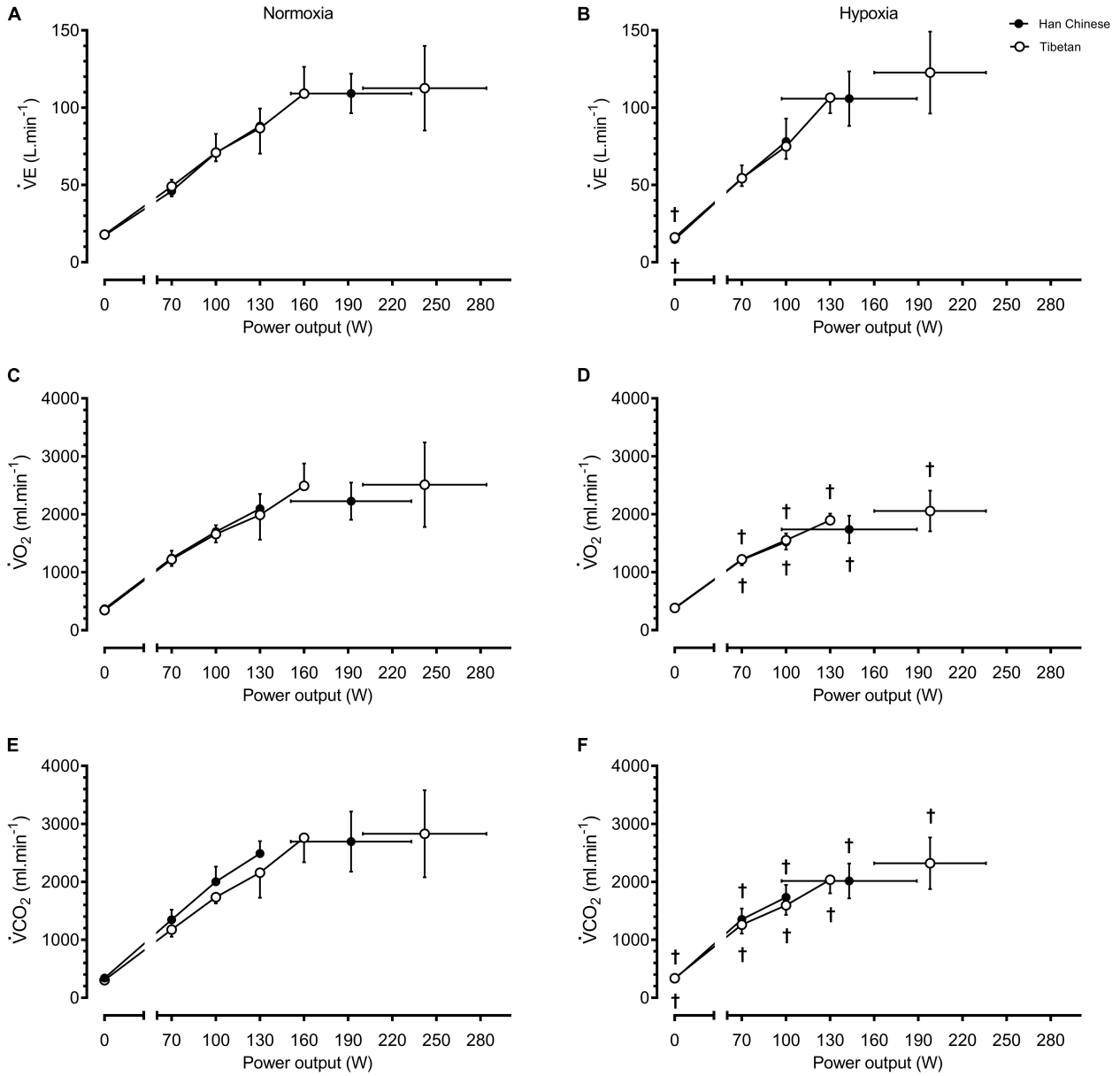

FIGURE 3 | Ventilation and metabolic responses during baseline, step-incremental cycling and maximal exertion (MAX) in Han Chinese and Tibetans. Left panel, normoxia: Right panel, hypoxia (equivalent of ∼5,000 m altitude). $\dot{V}E$, pulmonary ventilation; $\dot{V}O_2$, O_2 uptake; $\dot{V}CO_2$, expired CO_2. *different from Han Chinese, $p < 0.05$; †different from normoxia I, $p < 0.05$. Data expressed mean ± SD.

No group effects were observed in resting muscle NIRS signals ($p > 0.05$, **Table 2**).

During step-incremental cycling, hypoxia lowered muscle [O_2Hb] by ∼3.1 μM in both groups ($F = 10.7$, $d = 0.5$, $p < 0.001$), and elevated muscle [HHb] by ∼4.7 μM ($F = 15.1$, $d = 0.5$, $p < 0.001$), while muscle [totHb] was unchanged ($F = 2.6$, $p = 0.108$, **Figure 4**). This hypoxia effect on exercising muscle [O_2Hb] appeared to be limited to Tibetans (interaction: $F = 23.3$, $p < 0.001$). Hypoxia lowered muscle [O_2Hb] by ∼7.6 μM in Tibetans ($d = 1.0$, $p < 0.001$ vs. normoxia) but not in Han Chinese ($d = 0.3$, $p = 0.298$, **Figure 4**). As a result, muscle O_2Hb was selectively lower in Tibetans during hypoxic exercise (by ∼6.7 μM, $d = 0.9$, $p = 0.003$ vs. Han Chinese), but not during normoxic exercise ($d = 0.4$, $p = 0.240$, **Figure 4**).

We observed an interaction between hypoxia and group on muscle [totHb] ($F = 8.7$, $p = 0.004$). *Post hoc* analysis showed that hypoxia selectively increased muscle [totHb] in Han Chinese (by ∼4.6 μM, $d = 0.6$, $p = 0.003$), but not in Tibetans ($d = 0.2$, $p = 0.325$, **Figure 4**). Nevertheless, this increase did not result in a significant difference in muscle [totHb] between the groups during exercise in hypoxia ($d = 0.5$, $p = 0.117$). We found no significant group effects on muscle

TABLE 4 | Energy economy in Han Chinese and Tibetans during exercise at simulated sea-level and 5,000 m.

		Han Chinese		Tibetan		Main effects (*p*-values)		
n		**10**		**10**				
		Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**	**Condition**	**Group**	**Interaction**
EE ($J.s^{-1}$)	70 W	448 ± 46	440 ± 40	428 ± 40	435 ± 24	0.245	0.221	0.337
	100 W	626 ± 43	555 ± 50	592 ± 46	552 ± 42			
	130 W	773 ± 82	–	717 ± 149	681 ± 48			
	160 W	–	–	903 ± 136	–			
	Max	829 ± 133	643 ± 81†	924 ± 240	751 ± 129	**0.005**	0.958	0.238
GE (%)	70 W	14.9 ± 1.7	14.9 ± 1.8	16.5 ± 1.6	16.1 ± 1.4	0.129	0.397	0.494
	100 W	14.3 ± 1.1	16.6 ± 2.1	17.0 ± 1.4	18.2 ± 1.4			
	130 W	14.9 ± 1.8	–	19.1 ± 5.2	19.2 ± 1.2			
	160 W	–	–	18.2 ± 3.7	–			
	Max	23.1 ± 2.7	24.1 ± 4.2	26.7 ± 3.8*	26.6 ± 4.1*	0.120	**0.004**	0.823
EC ($kJ.L^{-1}$)	70 W	0.81 ± 0.09	0.83 ± 0.06	0.83 ± 0.08	0.82 ± 0.04	0.875	0.398	0.671
	100 W	0.85 ± 0.06	0.95 ± 0.09	0.87 ± 0.08	0.93 ± 0.07			
	130 W	0.90 ± 0.11	–	0.99 ± 0.28	0.99 ± 0.06			
	160 W	–	–	0.96 ± 0.21	–			
	Max	1.22 ± 0.15	1.26 ± 0.22	1.39 ± 0.18*	1.43 ± 0.23*	0.468	**0.034**	0.773

*EE, energy expenditure; GE, gross efficiency; EC, exercise economy. Values are mean ± SD. †different from normoxia ($p < 0.05$). *different from Han Chinese ($p < 0.05$). Bold text indicate significant main effect ($p < 0.05$).*

[HHb] during step-incremental cycling ($F = 0.1$, $p = 0.778$, **Figure 4**). No significant condition or group effects were observed on muscle tissue NIRS signals during recovery or at maximal effort ($p > 0.05$).

Cerebral Tissue Oxygenation and Hemodynamics

When compared to normoxia, hypoxia lowered resting cerebral [HHb] in both groups by ∼2.6 μM ($F = 10.3$, $d = 0.9$, $p = 0.005$) and [totHb] by ∼3.5 μM ($F = 6.9$, $d = 0.7$, $p = 0.017$), but had no effect on resting MCAv ($F = 1.1$, $p = 0.302$) or cerebral [O_2Hb] ($F = 1.0$, $p = 0.321$, **Table 2**). No group effects were observed in resting cerebral haemodynamics ($p > 0.05$, **Table 2**).

On average, hypoxia elevated cerebral [HHb] during step-incremental cycling by ∼3.7 μM in both Tibetan and Han Chinese ($F = 58.4$, $d = 1.8$), and lowered cerebral [O_2Hb] by ∼7.9 μM ($F = 67.8$, $d = 1.6$) and cerebral [totHb] by ∼4.1 μM ($F = 12.4$, $d = 0.6$, $p < 0.001$ for all, **Figure 5**). Hypoxia also lowered MCAv by ∼8.4 $cm.s^{-1}$ ($F = 29.6$, $d = 0.5$, $p < 0.001$), this reduction was greater in the Han Chinese group (interaction: $F = 8.0$, $p = 0.006$, **Figure 5**). In Han Chinese, hypoxia lowered MCAv by ∼12.8 $cm.s^{-1}$ during step-incremental cycling ($d = 0.7$, $p < 0.001$), while hypoxia only tended to lower MCAv in Tibetans by ∼4.1 $cm.s^{-1}$ ($d = 0.4$, $p = 0.055$). Tibetans had lower MCAv compared to Han Chinese in normoxia (by ∼15.3 $cm.s^{-1}$, $d = 0.8$, $p = 0.039$), while they were not significantly different in hypoxia ($d = 0.5$, $p = 0.360$, **Figure 5**). We did not observe any significant group effects on cerebral [O_2Hb] ($F = 0.1$, $p = 0.766$), [HHb] ($F = 2.1$, $p = 0.164$) or [totHb] during step-incremental cycling ($F = 0.6$, $p = 0.438$, **Figure 5**).

Hypoxia lowered cerebral [O_2Hb] during recovery, by ∼3.9 μM in both groups ($F = 7.3$, $d = 0.8$, $p = 0.011$ vs. normoxia) and cerebral [totHb] by ∼4.3 μM ($F = 8.6$, $d = 0.8$, $p = 0.006$), compared to normoxia but not MCAv ($F = 2.4$, $p = 0.140$) or cerebral [HHb] ($F = 1.2$, $p = 0.283$, **Figure 5**). We found no between-group differences in cerebral haemodynamics during recovery ($p > 0.05$, **Figure 5**).

At maximal effort, hypoxia lowered MCAv by ∼13.8 $cm.s^{-1}$ ($F = 17.6$, $d = 1.0$, $p < 0.001$), cerebral [O_2Hb] by ∼10.9 μM ($F = 54.5$, $d = 1.3$) and cerebral [totHb] by ∼6.9 μM ($F = 13.0$, $d = 0.8$), and elevated cerebral [HHb] by ∼4.1 μM ($F = 17.9$, $d = 1.0$, $p < 0.01$ for all vs. normoxia, **Figure 5**). We observed a group difference in cerebral [O_2Hb] ($F = 5.6$, $p = 0.031$), but only in normoxia (interaction: $F = 8.6$, $p = 0.010$). Compared to Han Chinese, cerebral [O_2Hb] was higher in Tibetans at maximal effort in normoxia by ∼8.9 μM ($d = 1.1$, $p = 0.001$), but not in hypoxia ($d = 0.0$, $p = 0.962$, **Figure 5**). Similarly, there was a significant group effect on cerebral [totHb] ($F = 5.4$, $p = 0.033$). As a result, hypoxia reduced cerebral [totHb] during maximal effort in Tibetans by ∼10.8 μM ($d = 1.2$, $p = 0.001$ vs. normoxia), but not in Han Chinese ($d = 0.4$, $p = 0.285$, **Figure 5**). No group effect was observed in cerebral [HHb] at maximal exercise ($F = 1.5$, $p = 0.235$).

DISCUSSION

The underlying mechanisms responsible for Tibetans' superior work capacity at altitude remains unclear. We compared cerebral and muscle tissue oxygenation responses to step-incremental cycling and at maximal exercise during ramp incremental cycling between Tibetans and Han Chinese in normobaric normoxia and hypobaric hypoxia in a pressure-regulated

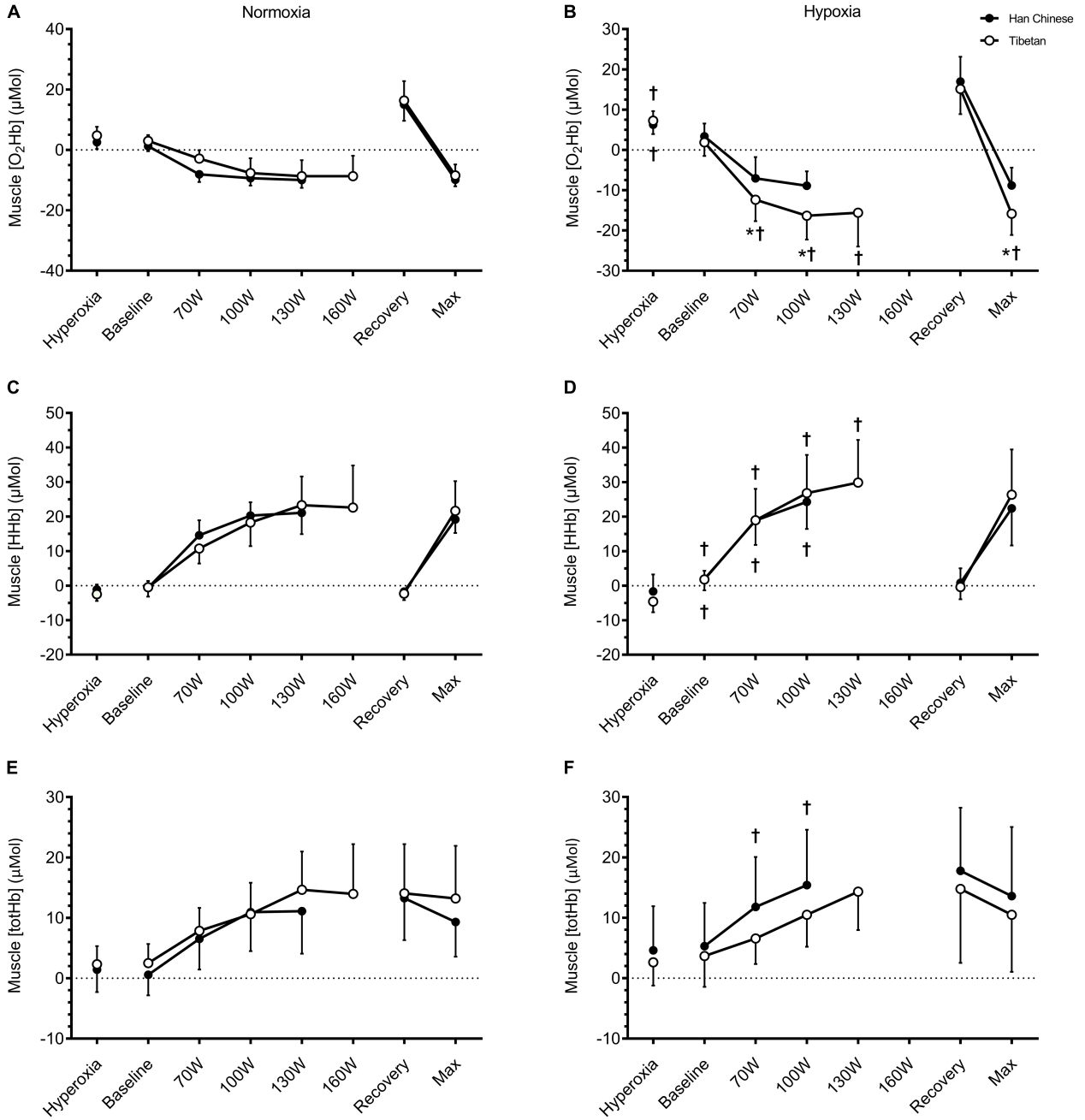

FIGURE 4 | Muscle tissue oxygenation of the vastus lateralis during hyperoxia, baseline, step-incremental cycling, recovery and ramp-incremental cycling to exhaustion (Max) in Han Chinese and Tibetans. Left panel, normoxia: Right panel, hypoxia (equivalent of ∼5,000 m altitude). *different from Han Chinese, $p < 0.05$; †different from normoxia, $p < 0.05$. Data expressed mean ± SD.

chamber, simulating altitudes equivalent to sea-level and 5,000 m altitude, respectively. Our main findings are that: (1) Irrespective of the condition, Tibetans consistently outperformed their Han Chinese counterparts and exhibited better economy at maximal exertion; (2) In normoxia, Tibetans displayed lower MCAv during submaximal exercise, yet achieved higher cerebral [O_2Hb] and [totHb] at maximal effort compared to Han Chinese; (3) In hypoxia, Tibetans displayed lower muscle [O_2Hb] compared to the Han Chinese, which was not mediated by any differences in SaO_2 or CaO_2; and (4) For a given workload, Tibetans exhibited greater muscle desaturation during exercise in hypoxia, but not in normoxia. It follows that during exercise in hypoxia Tibetans as compared to Han Chinese seem to defend their brain oxygenation over muscle oxygenation without any obvious cost to performance.

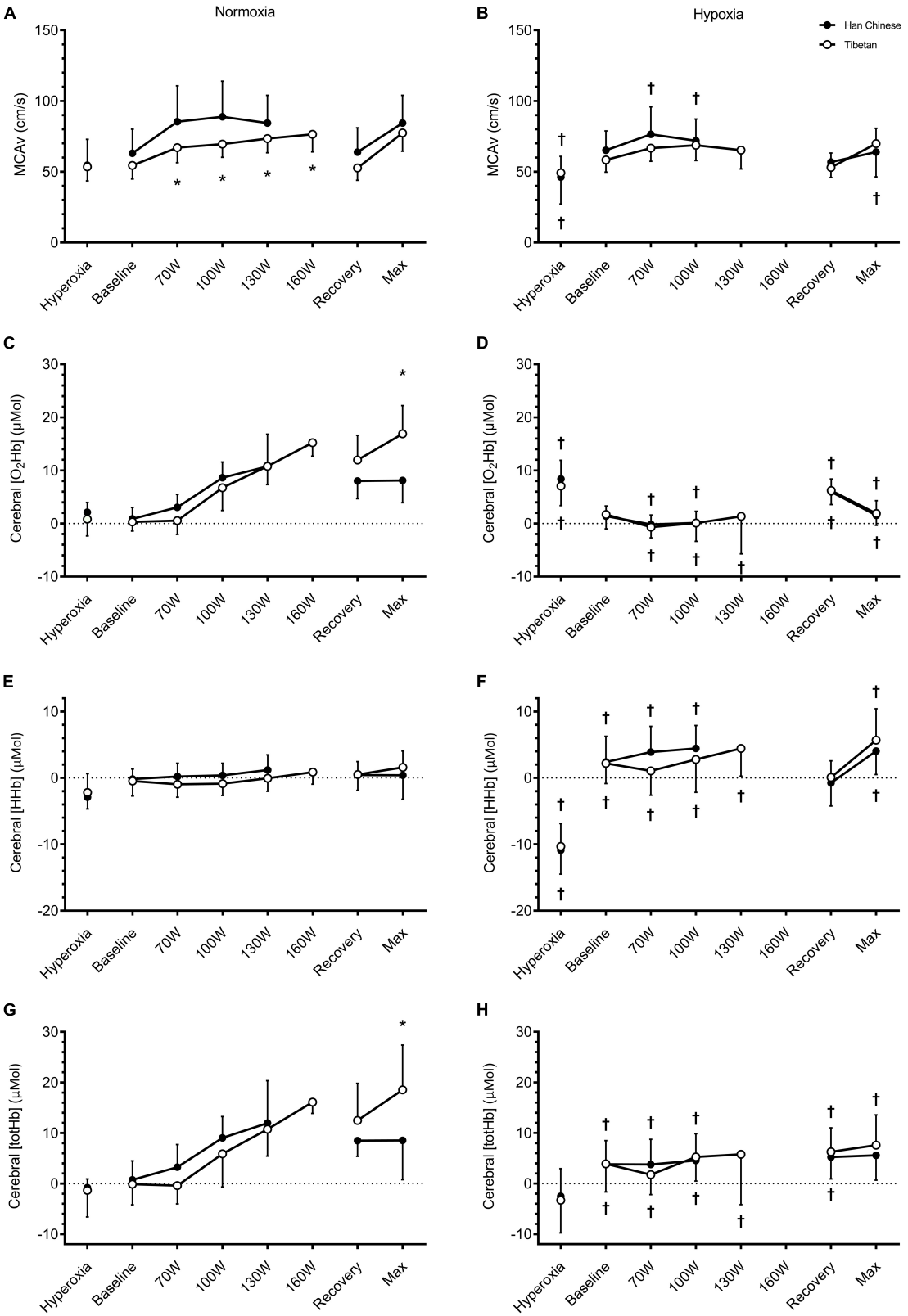

FIGURE 5 | Cerebral tissue oxygenation of the prefrontal cortex during hyperoxia, baseline, step-incremental cycling, recovery and ramp-incremental cycling to exhaustion (Max) in Han Chinese and Tibetans. Left panel, normoxia: Right panel, hypoxia (equivalent of ∼5,000 m altitude). *different from Han Chinese, $p < 0.05$; †different from normoxia, $p < 0.05$. Data expressed mean ± SD.

For a given workload, Subudhi et al. (2008) previously found comparable decreases in muscle [O_2Hb] between exercise at low altitude and at 4,300 m in lowlanders, while muscle [HHb] was increased at high altitude, suggesting greater O_2 extraction. Hypoxic training has been shown to enhance muscle [HHb] response to hypoxic exercise, while muscle [O_2Hb] response was unaffected (Wang et al., 2010). Our Han Chinese confirmed these observations by showing unchanged muscle [O_2Hb] responses to incremental exercise in hypobaric hypoxia when comparing with identical workloads in normoxia (**Figure 4**). Intriguingly, hypobaric hypoxia elicited a greater reduction in muscle [O_2Hb] in Tibetans during exercise, while the increase in [HHb] was unaffected (**Figure 4B**). Since both SaO_2 and CaO_2 were similar in Tibetans and Han Chinese during hypoxic exercise (**Table 3**), this would suggest similar O_2 extraction in Tibetans during hypoxic exercise (similar changes in [HHb]), but lower muscle perfusion (less [O_2Hb] and [totHb]). Our muscle NIRS data allude to lower muscle O_2 utilization in Tibetans for a given workload in hypoxia compared to Han Chinese. Furthermore, Tibetans achieved higher maximal workload despite similar $\dot{V}O_2$max in normoxia, and thus exhibited better exercise economy (**Table 4**). Our data support a previous study by Ge et al. (1994), who reported lower $\dot{V}O_2$ consumption in Tibetans for a given submaximal exercise load compared to well-acclimatized (∼3.2 years) Han Chinese at 4,700 m, along with greater maximal work at a lower $\dot{V}O_2$max. These findings allude to an enhanced muscle O_2 economy, presumably due to a metabolic adaptation in Tibetans. Intriguingly, this enhanced muscle O_2 economy appears to persist following descent to lower altitude (Marconi et al., 2005).

The underlying mechanism for this lower muscle perfusion and O_2 utilization in Tibetans is unclear. One possible explanation is a higher reliance on glucose oxidation in Tibetans during hypoxic exercise. Glucose oxidation generates higher number of high-Englert phosphate bonds per mole of O_2 (6.3 unit.mol O_2^{-1}) compared to fatty acid oxidation (4.1 unit.mol O_2^{-1}) (Kessler and Friedman, 1998). Therefore a higher reliance of glucose oxidation coupled with a lower reliance on fat oxidation increases the amount of ATP produced per molecule of O_2 consumed, thereby enhances work-to-O_2 ratio in the muscle (Hochachka et al., 1992; Hoppeler and Vogt, 2001). There is a predilection in Tibetans and Sherpas (a Nepalese ethnic group of Tibetan origin) toward carbohydrate oxidation and reduced reliance on intramyocellular lipids and lipid substrates (Hochachka et al., 1992; Kayser et al., 1996; Ge et al., 2015; Horscroft et al., 2017). The capacity of fatty acid oxidation is typically reduced at high altitude, mediated by decreased 3-hydroxyacyl-CoA dehydrogenase activity (Levett et al., 2012; Horscroft and Murray, 2014). Additional evidence for metabolic adaptation emerged from a genomic scan in Tibetan highlanders, where a haplotype of peroxisome proliferator-activated receptor α (PPARα) which was positively selected and associated with lower hematocrit (Simonson et al., 2010). PPARα encodes the nuclear peroxisome proliferator activated receptor α, which regulates fatty acid metabolism (Cullingford et al., 2002). At rest, we found acute hypoxic exposure selectively lowered RER in Han Chinese, presumably due to a shift toward fat utilization, while it was unchanged in Tibetans (**Table 2**). As reviewed by Gilbert-Kawai et al. (2014), our findings support a preferential use of carbohydrate-metabolism in Tibetans.

A potential explanation for the ability to extract oxygen to a similar extent despite reduced perfusion and saturation is a reduced muscle diffusion path for O_2 in Tibetans. In altitude-born Sherpas, Kayser et al. (1991) found muscle fiber cross-sectional area to be smaller, while capillary density was higher when compared to untrained lowlanders, but not different from that of fully acclimatized Caucasian climbers having spent two months at extreme altitude. This combination of smaller fiber cross-sectional area coupled with higher capillary density reduces the diffusion path for O_2. Since these muscle characteristics were similar between Sherpas and acclimatized Caucasian climbers, it is likely the result of adaptation to extreme altitude *per se*, rather than a unique feature of the Himalayan natives. In addition, lower mitochondrial densities have been observed in both high altitude and lowland-dwelling Sherpas and Tibetans, resulting in a higher maximal O_2 consumption-to-mitochondrial volume ratio (Kayser et al., 1991, 1996). Collectively, a shortened O_2 diffusion path and higher mitochondrial O_2 consumption would facilitate the higher muscle oxidative metabolism in Tibetans during hypoxic exercise.

Hyperventilation-induced hypocapnia during high-intensity exercise causes cerebral vasoconstriction, which can compromise cerebral O_2 delivery and decrease cerebral tissue oxygenation (Fan and Kayser, 2016). In Han Chinese, we observed a steady rise in MCAv during step-incremental cycling in normoxia, which began to decline during workloads above 100 W (**Figure 5A**), likely the result from hypocapnia (**Table 3**). In contrast, Tibetans did not display the expected decline in MCAv at higher exercise intensity in normoxia, despite similar $PaCO_2$ values (**Table 3**). Instead, MCAv, cerebral [O_2Hb] and [totHb] continued to rise in Tibetans during high intensity normoxic exercise (**Figure 5**). This difference in MCAv response resulted in higher cerebral [O_2Hb] and [totHb] in Tibetans at maximal exercise compared to Han Chinese. This finding supports a blunted MCAv response to hypocapnia in Tibetans during heavy intensity exercise. Together with their higher cardiac output, this enabled them to maintain higher prefrontal tissue oxygenation at maximal effort. Since cerebral tissue deoxygenation has been proposed as one of the limiting factors of exercise performance (Nybo and Rasmussen, 2007; Amann and Kayser, 2009), the ability to maintain higher cerebral tissue oxygenation in normoxia could partly account for the superior aerobic performance in Tibetans.

The effect of hypoxia on the cerebral perfusion response to exercise varies between populations. In Tibetans living at 3,658 m, Huang et al. (1992) found a greater increase in internal carotid artery (ICA) blood velocity and estimated

cerebral O_2 delivery during incremental exercise compared to Han Chinese. They found ICA blood velocity returned toward resting values at maximal exercise in the Han Chinese group, while ICA blood velocity remained elevated in Tibetans. During incremental exercise at simulated 5,000 m, we found no difference in MCAv and cerebral NIRS responses between Tibetan and Han Chinese (**Figure 5**). Compared to their normoxic values, hypoxia lowered MCAv during exercise by ∼23% in Han Chinese, while it only tended to lower it by ∼6% in Tibetans. As a result, the between-group difference in MCAv and cerebral NIRS responses to normoxic exercise were abolished during exercise in hypoxia. Together with the findings by Huang et al. (1992), our data indicates that Tibetans have a blunted cerebrovascular response to hypoxia compared to their Han Chinese counterparts, without adversely lowering cerebral tissue oxygenation.

We previously found higher SpO_2 (∼9%) and HR (∼13 b/min) in Tibetans during treadmill running in hypoxia compared to their Han Chinese counterparts (Kayser et al., 2019). Furthermore, iloprost inhalation improved hypoxic aerobic capacity in Han Chinese but not Tibetans, alluding to hypoxic pulmonary vasoconstriction and right ventricular function as limiting factors of hypoxic performance in Han. In the present study, we observed no between-group difference in SpO_2 or maximal HR during hypoxic cycling exercise (**Figure 2**). Given that maximal HR is typically higher during incremental treadmill running compared to incremental cycling in trained and untrained individuals, and is influenced by training mode and postural position [see Millet et al. (2009) for review], we attribute the discrepant HR and SpO_2 findings to the differences in exercise modality (cycling vs. treadmill running) and postural position (reclined and tilted vs. upright).

Methodological Considerations

There are several methodological considerations to be taken into account when interpreting our data. First, the PaO_2 values we obtained in normoxic conditions were higher than expected (∼130 mmHg, **Table 2**). There are two possible explanations for this. Firstly, the simulated normoxic condition was mildly hyperbaric due to instrumental/chamber setting error. However, independent measure of chamber pressure indicates this was unlikely the case. Alternatively, there was a leak from the O_2 masks used for the 100% O_2 breathing which resulted in FIO_2 being ∼0.22–0.24. This is the most likely explanation since we also observed slightly elevated PaO_2 in the hypobaric hypoxic condition (**Table 2**). Accordingly, our findings should be interpreted as mild hyperoxia rather than normoxia. While the cause of the high PaO_2 values is perplexing, we believe this does not affect our between-group comparisons since both Han Chinese and Tibetans exhibited high PaO_2 (**Table 2**).

Second, muscle oximetry based on NIRS signals provides non-invasive and region-specific information of changes in [O_2Hb] and [HHb] of the skeletal muscle tissue at rest and during exercise [see (Grassi and Quaresima, 2016; Perrey and Ferrari, 2017) for reviews], but there is much debate concerning the relative contributions of Hb and Mb to the muscle NIRS signals. At rest, Mb may contribute as much as ∼80% of the NIRS signal (Tran et al., 1999; Marcinek et al., 2007; Bendahan et al., 2017), but this value varies in relation to blood flow (as during exercise) and ambient PO_2 (i.e., hypoxia) Spires et al. (2011). Given that Mb plays a crucial role in hypoxic-tolerance in deep-diving mammals (Fago and Jensen, 2015), and is expressed in greater proportions in Tibetans (Gelfi et al., 2004), it is possible the accentuated muscle tissue deoxygenation in Tibetans is the result of greater Mb deoxygenation during hypoxic exercise.

Finally, we recruited a small sample (n = 10 in each group) of recreationally active individuals. Tibetans in this study consistently outperformed Han Chinese by ∼32% under both normoxic and hypoxic conditions, suggesting better efficiency during cycling in Tibetans. Wang et al. (2010) found hypoxic training does not affect muscle [O_2Hb] response to hypoxic exercise nor cerebral NIRS parameters during normoxic exercise, despite a ∼50% improvement in maximal workload (+22 $ml.min^{-1}.kg$ $\dot{V}O_2max$) in their participants. Recently, Caen et al. (2019) showed aerobic training increased the amplitude of muscle totHb and HHb responses during incremental exercise in normoxia, indicating improved O_2 availability and muscle O_2 extraction with improved fitness. Meanwhile, we found comparable muscle tissue oxygenation responses between Han Chinese and Tibetans during incremental exercise in normoxia, and lower muscle O_2Hb and totHb in Tibetans during hypoxic exercise. We contend that the between-group differences in muscle tissue oxygenation during incremental exercise is due to ethnicity rather than fitness.

CONCLUSION

We found distinct differences between Tibetans and Han Chinese in muscle and brain tissue oxygenation changes during exercise. We found Tibetans exhibited a blunted cerebrovascular response to hypocapnia during normoxic exercise. This combined with a higher heart rate (and cardiac output) enabled them to maintain a higher cerebral tissue oxygenation at maximal effort compared to Han Chinese. During hypoxic exercise, we found evidence of greater muscle tissue deoxygenation in Tibetans for a given workload, which we interpret as enhanced muscle O_2 extraction. Tibetans consistently outperformed their Han Chinese counterpart in both normoxic and hypoxic conditions, exhibiting better energy economy at exercise exertion. For the first time, our data demonstrate that Tibetans can maintain higher cerebral tissue oxygenation during maximal normoxic exercise and enhance muscle O_2 utilization during hypoxic exercise. Whether these muscular and cerebrovascular responses account for their superior aerobic performance warrants further investigation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Oregon Institutional Review Board and the Qinghai High Altitude Medical Science Institutional Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-LF, TW, AL, and BK contributed to the conception and design of the study. J-LF, TW, LN, WLB, AL, and BK performed the data collection. J-LF, AL, and BK carried out the analysis, carried out the interpretation of the data, and contributed to the revision of the manuscript. J-LF drafted the manuscript and prepared the figures. All authors approved the final version of the manuscript.

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REFERENCES

Allemann, Y., Hutter, D., Lipp, E., Sartori, C., Duplain, H., Egli, M., et al. (2006). Patent foramen ovale and high-altitude pulmonary edema. *JAMA* 296, 2954–2958. doi: 10.1001/jama.296.24.2954

Amann, M., Eldridge, M. W., Lovering, A. T., Stickland, M. K., Pegelow, D. F., and Dempsey, J. A. (2006). Arterial oxygenation influences central motor output and exercise performance via effects on peripheral locomotor muscle fatigue in humans. *J. Physiol.* 575 (Pt 3), 937–952.

Amann, M., and Kayser, B. (2009). Nervous system function during exercise in hypoxia. *High Alt. Med. Biol.* 10, 149–164. doi: 10.1089/ham.2008.1105

Amann, M., Romer, L. M., Subudhi, A. W., Pegelow, D. F., and Dempsey, J. A. (2007). Severity of arterial hypoxaemia affects the relative contributions of peripheral muscle fatigue to exercise performance in healthy humans. *J. Physiol.* 581 (Pt 1), 389–403.

Bendahan, D., Chatel, B., and Jue, T. (2017). Comparative NMR and NIRS analysis of oxygen-dependent metabolism in exercising finger flexor muscles. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 313, R740–R753. doi: 10.1152/ajpregu.00203.2017

Brenner, R., Pratali, L., Rimoldi, S. F., Murillo Jauregui, C. X., Soria, R., Rexhaj, E., et al. (2015). Exaggerated pulmonary hypertension and right ventricular dysfunction in high-altitude dwellers with patent foramen ovale. *Chest* 147, 1072–1079. doi: 10.1378/chest.14-1353

Caen, K., Vermeire, K., Pogliaghi, S., Moerman, A., Niemeijer, V., Bourgois, J. G., et al. (2019). Aerobic interval training impacts muscle and brain oxygenation responses to incremental exercise. *Front. Physiol.* 10:1195. doi: 10.3389/fphys.2019.01195

Cohen, J. (1977). *Statistical Power Analysis for the Behavioral Sciences*. New York, NY: Academic Press.

Cullingford, T. E., Dolphin, C. T., and Sato, H. (2002). The peroxisome proliferator-activated receptor alpha-selective activator ciprofibrate upregulates expression of genes encoding fatty acid oxidation and ketogenesis enzymes in rat brain. *Neuropharmacology* 42, 724–730. doi: 10.1016/s0028-3908(02)00014-x

Duncan, A., Meek, J. H., Clemence, M., Elwell, C. E., Tyszczuk, L., Cope, M., et al. (1995). Optical pathlength measurements on adult head, calf and forearm and the head of the newborn infant using phase resolved optical spectroscopy. *Phys. Med. Biol.* 40, 295–304. doi: 10.1088/0031-9155/40/2/007

Elliott, J. E., Laurie, S. S., Kern, J. P., Beasley, K. M., Goodman, R. D., Kayser, B., et al. (2015). AltitudeOmics: impaired pulmonary gas exchange efficiency and blunted ventilatory acclimatization in humans with patent foramen ovale after 16 days at 5,260 m. *J. Appl. Physiol. (1985)* 118, 1100–1112. doi: 10.1152/japplphysiol.00879.2014

Erzurum, S. C., Ghosh, S., Janocha, A. J., Xu, W., Bauer, S., Bryan, N. S., et al. (2007). Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17593–17598. doi: 10.1073/pnas.0707462104

Fago, A., and Jensen, F. B. (2015). Hypoxia tolerance, nitric oxide, and nitrite: lessons from extreme animals. *Physiology (Bethesda)* 30, 116–126. doi: 10.1152/physiol.00051.2014

Fan, J. L., and Kayser, B. (2016). Fatigue and exhaustion in hypoxia: the role of cerebral oxygenation. *High Alt. Med. Biol.* 17, 72–84. doi: 10.1089/ham.2016.0034

Foster, G. E., Ainslie, P. N., Stembridge, M., Day, T. A., Bakker, A., Lucas, S. J., et al. (2014). Resting pulmonary haemodynamics and shunting: a comparison of sea-level inhabitants to high altitude Sherpas. *J. Physiol.* 592(Pt 6), 1397–1409. doi: 10.1113/jphysiol.2013.266593

Ge, R. L., Chen, Q. H., Wang, L. H., Gen, D., Yang, P., Kubo, K., et al. (1994). Higher exercise performance and lower VO2max in tibetan than han residents at 4,700 m altitude. *J. Appl. Physiol.* 77, 684–691.

Ge, R. L., Simonson, T. S., Gordeuk, V., Prchal, J. T., and McClain, D. A. (2015). Metabolic aspects of high-altitude adaptation in Tibetans. *Exp. Physiol.* 100, 1247–1255. doi: 10.1113/EP085292

Gelfi, C., De Palma, S., Ripamonti, M., Eberini, I., Wait, R., Bajracharya, A., et al. (2004). New aspects of altitude adaptation in Tibetans: a proteomic approach. *FASEB J.* 18, 612–614. doi: 10.1096/fj.03-1077fje

Gilbert-Kawai, E. T., Milledge, J. S., Grocott, M. P., and Martin, D. S. (2014). King of the mountains: tibetan and Sherpa physiological adaptations for life at high altitude. *Physiology (Bethesda)* 29, 388–402. doi: 10.1152/physiol.00018.2014

Grassi, B., and Quaresima, V. (2016). Near-infrared spectroscopy and skeletal muscle oxidative function in vivo in health and disease: a review from an exercise physiology perspective. *J. Biomed. Opt.* 21, 091313. doi: 10.1117/1.JBO.21.9.091313

Hochachka, P. W., Stanley, C., McKenzie, D. C., Villena, A., and Monge, C. (1992). Enzyme mechanisms for pyruvate-to-lactate flux attenuation: a study of Sherpas, Quechuas, and hummingbirds. *Int. J. Sports Med.* 13(Suppl. 1), S119–S122. doi: 10.1055/s-2007-1024613

Hoppeler, H., and Vogt, M. (2001). Muscle tissue adaptations to hypoxia. *J. Exp. Biol.* 204(Pt 18), 3133–3139.

Horscroft, J. A., Kotwica, A. O., Laner, V., West, J. A., Hennis, P. J., Levett, D. Z. H., et al. (2017). Metabolic basis to Sherpa altitude adaptation. *Proc. Natl. Acad. Sci. U.S.A.* 114, 6382–6387. doi: 10.1073/pnas.1700527114

Horscroft, J. A., and Murray, A. J. (2014). Skeletal muscle energy metabolism in environmental hypoxia: climbing towards consensus. *Extrem. Physiol. Med.* 3:19. doi: 10.1186/2046-7648-3-19

Huang, S. Y., Sun, S., Droma, T., Zhuang, J., Tao, J. X., McCullough, R. G., et al. (1992). Internal carotid arterial flow velocity during exercise in Tibetan and Han residents of Lhasa (3,658 m). *J. Appl. Physiol.* 73, 2638–2642.

Kayser, B., Fan, J. L., Nan, L., Bang, W. L., Bianba, and Wu, T. (2019). Iloprost improves running performance at 5,000m in Han but not in Tibetans. *Curr. Issues Sport Sci.* 4:002. doi: 10.15203/CISS_2019.002

Kayser, B., Hoppeler, H., Claassen, H., and Cerretelli, P. (1991). Muscle structure and performance capacity of Himalayan Sherpas. *J. Appl. Physiol. (1985)* 70, 1938–1942.

Kayser, B., Hoppeler, H., Desplanches, D., Marconi, C., Broers, B., and Cerretelli, P. (1996). Muscle ultrastructure and biochemistry of lowland Tibetans. *J. Appl. Physiol. (1985)* 81, 419–425.

Kelman, G. R., and Nunn, J. F. (1966). Nomograms for correction of blood PO2, PCO2, pH, and base excess for time and temperature. *J. Appl. Physiol.* 21, 1484–1490.

Kessler, G., and Friedman, J. (1998). Metabolism of fatty acids and glucose. *Circulation* 98:1351.

Kjaer, M., Hanel, B., Worm, L., Perko, G., Lewis, S. F., Sahlin, K., et al. (1999). Cardiovascular and neuroendocrine responses to exercise in hypoxia during impaired neural feedback from muscle. *Am. J. Physiol.* 277(1 Pt 2), R76–R85.

Levett, D. Z., Radford, E. J., Menassa, D. A., Graber, E. F., Morash, A. J., Hoppeler, H., et al. (2012). Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *FASEB J.* 26, 1431–1441. doi: 10.1096/fj.11-197772

Lovering, A. T., Stickland, M. K., Amann, M., O'Brien, M. J., Hokanson, J. S., and Eldridge, M. W. (2011). Effect of a patent foramen ovale on pulmonary gas exchange efficiency at rest and during exercise. *J. Appl. Physiol. (1985)* 110, 1354–1361. doi: 10.1152/japplphysiol.01246.2010

Marcinek, D. J., Amara, C. E., Matz, K., Conley, K. E., and Schenkman, K. A. (2007). Wavelength shift analysis: a simple method to determine the contribution of hemoglobin and myoglobin to in vivo optical spectra. *Appl. Spectrosc.* 61, 665–669. doi: 10.1366/000370207781269819

Marconi, C., Marzorati, M., and Cerretelli, P. (2006). Work capacity of permanent residents of high altitude. *High Alt. Med. Biol.* 7, 105–115. doi: 10.1089/ham.2006.7.105

Marconi, C., Marzorati, M., Sciuto, D., Ferri, A., and Cerretelli, P. (2005). Economy of locomotion in high-altitude Tibetan migrants exposed to normoxia. *J. Physiol.* 569(Pt 2), 667–675. doi: 10.1113/jphysiol.2005.094979

Millet, G. P., Vleck, V. E., and Bentley, D. J. (2009). Physiological differences between cycling and running: lessons from triathletes. *Sports Med.* 39, 179–206. doi: 10.2165/00007256-200939030-00002

Millet, G. Y., Muthalib, M., Jubeau, M., Laursen, P. B., and Nosaka, K. (2012). Severe hypoxia affects exercise performance independently of afferent feedback and peripheral fatigue. *J. Appl. Physiol. (1985)* 112, 1335–1344. doi: 10.1152/japplphysiol.00804.2011

Moseley, L., and Jeukendrup, A. E. (2001). The reliability of cycling efficiency. *Med. Sci. Sports Exerc.* 33, 621–627. doi: 10.1097/00005768-200104000-00017

Nybo, L., and Rasmussen, P. (2007). Inadequate cerebral oxygen delivery and central fatigue during strenuous exercise. *Exerc. Sport Sci. Rev.* 35, 110–118.

Peltonen, J. E., Paterson, D. H., Shoemaker, J. K., Delorey, D. S., Dumanoir, G. R., Petrella, R. J., et al. (2009). Cerebral and muscle deoxygenation, hypoxic ventilatory chemosensitivity and cerebrovascular responsiveness during incremental exercise. *Respir. Physiol. Neurobiol.* 169, 24–35.

Perrey, S., and Ferrari, M. (2017). Muscle oximetry in sports science: a systematic review. *Sports Med.* 48, 597–616. doi: 10.1007/s40279-017-0820-1

Rasmussen, P., Dawson, E. A., Nybo, L., van Lieshout, J. J., Secher, N. H., and Gjedde, A. (2007). Capillary-oxygenation-level-dependent near-infrared spectrometry in frontal lobe of humans. *J. Cereb. Blood Flow Metab.* 27, 1082–1093. doi: 10.1038/sj.jcbfm.9600416

Rasmussen, P., Nielsen, J., Overgaard, M., Krogh-Madsen, R., Gjedde, A., Secher, N. H., et al. (2010). Reduced muscle activation during exercise related to brain oxygenation and metabolism in humans. *J. Physiol.* 588(Pt 11), 1985–1995.

Rupp, T., and Perrey, S. (2009). Effect of severe hypoxia on prefrontal cortex and muscle oxygenation responses at rest and during exhaustive exercise. *Adv. Exp. Med. Biol.* 645, 329–334. doi: 10.1007/978-0-387-85998-9_49

Severinghaus, J. W. (1966). Blood gas calculator. *J. Appl. Physiol.* 21, 1108–1116.

Simonson, T. S., Yang, Y., Huff, C. D., Yun, H., Qin, G., Witherspoon, D. J., et al. (2010). Genetic evidence for high-altitude adaptation in Tibet. *Science* 329, 72–75. doi: 10.1126/science.1189406

Smith, K. J., and Billaut, F. (2010). Influence of cerebral and muscle oxygenation on repeated-sprint ability. *Eur. J. Appl. Physiol.* 109, 989–999. doi: 10.1007/s00421-010-1444-4

Spires, J., Lai, N., Zhou, H., and Saidel, G. M. (2011). Hemoglobin and myoglobin contributions to skeletal muscle oxygenation in response to exercise. *Adv. Exp. Med. Biol.* 701, 347–352. doi: 10.1007/978-1-4419-7756-4_47

Subudhi, A. W., Dimmen, A. C., and Roach, R. C. (2007). Effects of acute hypoxia on cerebral and muscle oxygenation during incremental exercise. *J. Appl. Physiol.* 103, 177–183.

Subudhi, A. W., Lorenz, M. C., Fulco, C. S., and Roach, R. C. (2008). Cerebrovascular responses to incremental exercise during hypobaric hypoxia: effect of oxygenation on maximal performance. *Am. J. Physiol. Heart Circ. Physiol.* 294, H164–H171. doi: 10.1152/ajpheart.01104.2007

Sullivan, G. M., and Feinn, R. (2012). Using Effect Size-or Why the P Value Is Not Enough. *J Grad Med Educ* 4, 279–282. doi: 10.4300/JGME-D-12-00156.1

Tran, T. K., Sailasuta, N., Kreutzer, U., Hurd, R., Chung, Y., Mole, P., et al. (1999). Comparative analysis of NMR and NIRS measurements of intracellular PO2 in human skeletal muscle. *Am. J. Physiol.* 276(6 Pt 2), R1682–R1690.

Vogiatzis, I., Louvaris, Z., Habazettl, H., Athanasopoulos, D., Andrianopoulos, V., Cherouveim, E., et al. (2011). Frontal cerebral cortex blood flow, oxygen delivery and oxygenation during normoxic and hypoxic exercise in athletes. *J. Physiol.* 589(Pt 16), 4027–4039. doi: 10.1113/jphysiol.2011.210880

Wang, J. S., Wu, M. H., Mao, T. Y., Fu, T. C., and Hsu, C. C. (2010). Effects of normoxic and hypoxic exercise regimens on cardiac, muscular, and cerebral hemodynamics suppressed by severe hypoxia in humans. *J. Appl. Physiol. (1985)* 109, 219–229. doi: 10.1152/japplphysiol.00138.2010

Wu, T., and Kayser, B. (2006). High altitude adaptation in Tibetans. *High Alt. Med. Biol.* 7, 193–208. doi: 10.1089/ham.2006.7.193

Wu, T., Li, S., and Ward, M. P. (2005). Tibetans at extreme altitude. *Wilderness Environ. Med.* 16, 47–54.

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Cardiovascular Risk Is Increased in Miner's Chronic Intermittent Hypobaric Hypoxia Exposure From 0 to 2,500 m?

*Andrés Pedreros-Lobos[1], Rodrigo Calderón-Jofré[1,2], Daniel Moraga[3] and Fernando A. Moraga[1]**

[1]*Laboratorio de Fisiología, Hipoxia y Función Vascular, Departamento de Ciencias Biomédicas, Facultad de Medicina, Universidad Católica del Norte, Coquimbo, Chile,* [2]*Departamento de Ciencias Básicas, Universidad Central La Serena, La Serena, Chile,* [3]*Departamento de Medicina, Facultad de Ciencias de la Salud, Universidad de Tarapacá, Arica, Chile*

Over the past 40 years, mining activities in Chile have relocated miners who normally live at sea level to work at high altitudes. This results in a form of chronic intermittent hypobaric hypoxia (CIHH) characterized by alternating periods of work at high altitude and rest periods at sea level. Previous studies performed in our laboratory showed that aerobic capacity is reduced at 3,800 m, even when oxygen content is maintained. Our study aimed to determine the corporal composition, food intake, maximum oxygen uptake, and concentration of high sensitivity C reactive protein (*hs*CRP) in an acclimatized miner population that work from 0 to 2,500 m with CIHH exposure over 4 years. All miners recruited for our study were operators of heavy trucks with CIHH for over 4 years (shiftwork 7*7 days), and our experimental population was composed of 54 miners at sea level, 61 at 1,600 m, and 38 at 2,500 m. All evaluations were performed on the 3rd or 4th day of diurnal shiftwork. To determine corporal composition, we measured weight and height (to calculate body mass index, BMI), skinfolds (to calculate body fatty, BF), and waist circumference (WC); maximal aerobic capacity was evaluated using a ramp-incremental cycling to exhaustion protocol and a venous blood sample before the exercise test to measure (*hs*CRP) *via* an ELISA test. We found higher values of BMI, BF, and WC, in the miners' population but observed no significant difference between populations. We found a decrease in VO_2 of 11.6% at 1,600 m and 25.9% at 2,500 m compared to miners at sea level. An increase in (*hs*CRP) at 1,600 and 2,500 m regards sea level. We observed a high prevalence of overweight and obese subjects, which was related to the *ad libitum* availability of food and low physical activity (sedentarism). We found that work capacity was maintained despite a decreased VO_2 max at moderate altitude. However, overweight and obesity support an increased risk of cardiometabolic disease in miner's which is unrelated to altitude. In contrast, an increased *hs*CRP level could be associated with increased inflammatory mechanisms at 1,600 and 2,500 m.

Keywords: aerobic capacity, high sensitivity C reactive protein, corporal composition, cardiovascular risk, workers at high altitude, chronic intermittent hypobaric hypoxia

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****Correspondence:***
Fernando A. Moraga
fmoraga@ucn.cl

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INTRODUCTION

Over the last 40 years, mining activity in Chile has relocated miners that normally live at low altitudes (<1,000 m) to work at high altitudes (>3,000 m). This shift is called the "Chilean model of Chronic Intermittent Hypobaric Hypoxia (CIHH) exposure," which is characterized by alternating periods of work at high altitude and rest periods at low altitude (Richalet et al., 2002; Moraga et al., 2018).

Exposure to high altitude limits oxygen diffusion within the lungs, reducing oxygen transport into muscle and thereby affecting work capacity (Calbet and Lundby, 2009). However, exposure studies on maximal aerobic capacity in this model are scarce. Initial studies with CIHH miners began with a prospective study of CIHH exposure at a high altitude (4,500 m) for 31 months; where an inverse relationship between exposure time with decreased physical performance was observed (Richalet et al., 2002). A second study performed in soldiers with CIHH exposure for 6 months at a high altitude (3,550 m) showed a tendency for maximal aerobic capacity (VO_2 max) to decrease at this altitude but oxygen transport capacity was maintained (Prommer et al., 2007). A third study performed on miners acclimatized to CIHH for a long period (7–36 months) showed that the maintained oxygen transport is explained by an increased hemoglobin concentration alongside increased intensity, reaching the same intensity (Watts) at sea level and high altitude (Moraga et al., 2018). Furthermore, a study performed in healthy miners, a population with moderate physical activity at high altitude, showed that work capacity (intensity) is maintained despite the reduced oxygen consumption, supporting the notion that increased work efficiency occurs during maximal exercise at high altitude (Moraga et al., 2019).

Several studies described an increasing prevalence of weight gain and obesity along with other cardiovascular risk factors in native highlanders (Shah et al., 2004). A series of antecedents showed that obesity not only predisposes people to insulin resistance and diabetes but also contributes to atherogenic dyslipidemia (see reviewed by Libby et al., 2002). Additionally, adipose tissue can also synthesize cytokines such as TNF-∝ and IL-6 (Yudkin et al., 1999) thus obesity itself promotes inflammation and potentiates atherogenesis.

Growing antecedents reported in the literature indicate that elevated circulating inflammatory markers, such as C-reactive protein (CRP), predict coronary events, stroke, and progression of peripheral disease independent of the severity of atherosclerotic or ischemic events (Pepys and Hirschfield, 2003). Additionally, two studies have previously shown increased levels of IL-6 and CRP at high altitude compared to controls by two different ascent types (passive or active) at 4,559 m (Hartmann et al., 2000; Bailey et al., 2004). Hypoxia at high altitudes could be considered a new inflammatory stimulus below the expected range for inflammatory diseases, such as acute mountain sickness or high-altitude pulmonary edema (HAPE; Bailey et al., 2004). We evaluate whether an increase in work efficiency could be related to increased cardiovascular risk in miners that work at sea level and acclimatized mine workers exposed to moderate CIHH at 1,600 and 2,500 m.

SUBJECTS, MATERIALS, AND METHODS

A cross-sectional study and descriptive scope were carried out in 153 male miners with more than 4 years of experience. They undergo shift work characterized by 7 days of work followed by 7 days of rest. All miners work for the same company, which has operations at all three altitude levels (sea level, 1,600, and 2,500 m). Our population was composed of heavy truck operators where 54 miners worked at sea level, 61 at 1,600 m, and 38 at 2,500 m. Evaluations were performed on the 3rd and 4th day of diurnal shiftwork at sea level or high altitude. Protocols used in this study followed the International Ethical Guidelines (according to the Helsinki declaration) and were approved by the Ethics Committee of the Facultad de Medicina, Universidad Católica del Norte, Chile, and the Medical Director of the mining company. All volunteers were informed of the possible risks and discomfort involved before giving their signed consent to participate.

Corporal Composition

Our anthropometric evaluation considered body weight (BW, kg) and height (cm) using a scale balance (SECA model 767) to calculate body mass index (BMI, BMI = kg/m^2). Waist circumference (WC, cm) was measured using inextensible metric tape (SECA model 201). Body fat mass was determined by measuring four skinfolds: bicipital, tricipital, subscapular, and suprailiac (Lange skinfold, Cambridge, Maryland) percentage of adipose (Fatty body, %) was calculated using the protocol described by Durnin and Womersley (1974); and, finally, we calculated fat mass (FM, kg) as FM = (BW*FB)/100 and lean body mass (LBM, kg) as LBM = BW − FM. All anthropometrical measurements were made by the same evaluator. **Table 1** shows the classifications of cardiovascular risk concerning BMI, WC, body fat (%), and LBM (kg; Bray, 1998; Jensen et al., 2013; Macek et al., 2020). After evaluation of corporal composition, we measured oxygen saturation (SpO2, %) and heart rate (HR, bpm) by pulse oximetry (model 7500FO Nonin) and systolic arterial pressure (SAP) and diastolic arterial pressure (DAP) using a cardiorespiratory monitor (model BM3, Bionet).

TABLE 1 | Reference values and classification criteria of cardiovascular risk.

BMI (kg/m^2)		
Low	<18.5	Jensen et al., 2013
Normal	18.5–24.9	
Overweight	>24.9 and <29.9	
Obesity	>30	
BF (%)		
Normal	>12 and <20	Bray 1998
Overweight	>20 and <25	
Obesity	>25	
WC (cm)		
Normal	<94	Macek et al., 2020
Elevated	>94	
***hs*CRP (mg/L)**		
Low	<1	Libby et al., 2002
Moderated	>1 and >3	
High	>3	

Determination of the Energetic Balance

Evaluation of Dietary Intake

We assessed dietary intake with a standardized 24 h recall food survey (Olivares et al., 2007). The 24 h recall is a method employed to assess the type of food and the quantities consumed in the last 24 h. The nutritional contribution obtained from the survey allowed us to quantify the food intake and calculate the nutritional contribution of this food, which we then compared to a standardized table of recommended nutritional intake for the Chilean population (Zacarías et al., 2018). The dietary contribution values were expressed in Kilocalories (Kcal). All surveys were carried out by a nutritionist (AP-L) with experience in the use of this instrument.

Estimation of Energetic Expenditure

In our study, we used FAO/WHO/UNU equations (2001) to estimate the basal metabolic rest (BMR). We calculated total energetic expenditure (EET) using the following equation; EET = BMR × physical activity index. We defined a physical activity index using three levels according to lifestyle type which had persisted longer than their job occupation: sedentary (mean 1.55), moderate (mean 1.76), and active (mean 2.1; FAO/WHO/UNU, 2004; Westerterp, 2017). Physical activity in the workplace at sea level, 1,600, and 2,500 m was evaluated indirectly by using a IPAQs survey (International Physical Activity Questionnaire short; Ainsworth et al., 2000) in the morning of the 4th day, previous to the maximal aerobic capacity test.

Measurement of *hs*CRP

We collected blood samples from the brachial vein before entering the shift (between 07:00 and 08:00 am) on day 3 at the indicated altitude. Blood samples were centrifuged immediately after collection (3,000 rpm for 20 min) and the plasma fraction was frozen at −80°C until analysis. To measure high sensitive CRP (*hs*CRP) levels, an ELISA test was performed in duplicate using a commercial kit for serum and plasma (Human CRP/CRP Quantikine ELISA Kit; catalog number: DCRP00; R&D Systems, Minneapolis, MN 55413). **Table 1** shows the CV risk criteria according to the plasma level of *hs*CRP.

Maximal Aerobic Capacity

The exercise test was performed on the 4th day on a cycle ergometer (Model Corival, Lode) where oxygen consumption and ventilation (VE) variables were measured using a metabolic cart (Ultima CPX, Medgraphics, St. Paul, Minnesota, United States) calibrated before each test according to the manufacturer's instructions with high-grade calibration gases (purchased to INDURA, Chile). Respiratory variables were analyzed breath-by-breath in real-time and averaged 5 s during all tests. We also assessed transcutaneous arterial saturation (SpO2, %) and HR (bpm) by a pulse oximeter (7500FO Nonin Medical, Inc., United States) with the sensor placed on an ear lobe (8000Q2 Nonin Medical, Inc., United States). To measure maximum oxygen consumption (VO_2 max), we performed a ramp-incremental cycling to exhaustion protocol followed by a 10-min rest period seated on the ergometer. Each participant was instructed to begin cycling at 0 Watts, maintaining a cadence of 70 rpm. The work rate was increased by 0.5 Watts/s (or equivalent to 30 Watts/min) thereafter until the participant reached voluntary exhaustion. We considered VO_2 max when participants reached values over 85% of the estimated max load.

Statistical Analysis

Values presented in tables were expressed as the mean ± SD. Non-parametrical variables obtained in our study, such as percentages (%) were analyzed by proportional tests. Comparison of parametrical variables was analyzed using one-way ANOVA, and differences between different altitudes were evaluated by a Newman-Keuls test. Differences were considered statistically significant when values of $p < 0.05$. Pearson's correlation was also performed; the analysis was carried out to establish the association between *hs*CRP (dependent variable) with anthropometrical and cardiometabolic variables. All statistical analyses were performed with GraphPad Prism Software (version 5.03, GraphPad Software, Inc.).

RESULTS

Corporal Composition

Populations studied here showed no variation in BW, BMI, body fatty (BF), FM, LBM, and waist perimeter between the levels evaluated (**Table 2**). However, when analyzing populations based on the established criteria (**Table 1**), we observed BMI values in the overweight and obese range at sea level, 1,600, and 2,500 m of 80, 75, and 84%, respectively, and BF (%) values of 57.5, 57.5, and 58.5%, respectively. Additionally, we observed WC measurements which corresponded to a high risk of cardiovascular disease (over 94 cm) at sea level, 1,600, and 2,500 m of 43, 48.6, and 52.9%, respectively.

TABLE 2 | Anthropometrical characteristic of population that work at several altitudes.

	Altitude (m)		
	Sea level	**1,600**	**2,500**
n	54	61	38
Ages (years)	42.5 ± 10.5	37.2 ± 8.0	37.7 ± 9.1
Weight (kg)	78.3 ± 11.4	83.2 ± 11.2	81.2 ± 10.6
Height (m)	1.70 ± 0.07	1.73 ± 0.06	1.70 ± 0.05
BMI (kg/m^2)	27.2 ± 3.1	27.7 ± 2.7	28.1 ± 2.8
BF (%)	25.5 ± 4.2	26.9 ± 4.3	27.5 ± 4.4
BFM (kg)	21.7 ± 5.7	22.5 ± 5.3	21.8 ± 5.6
LBM (kg)	56.7 ± 7.0	60.7 ± 7.8	59.4 ± 6.5
WC (cm)	94.5 ± 8.9	95.1 ± 7.6	95.0 ± 8.0
Physical activity (%)			
Active	14.6	2.7	7.5
Moderate	17.1	21.6	24.0
Sedentary	68.3	75.7	68.5

Mean ± SD, Body mass index (BMI), Body fatty (BF), Body fatty mass (BFM), Lean body mass (LBM), and Waist circumference (WC).

Energetic Balance at Several Altitudes

Dietary Intake

The body composition results described above show that the population has risk factors associated with overweight and obesity, due to the dietary intake of volunteers being above normal dietary intake levels (**Table 3**). The population we studied also lived a largely sedentary lifestyle. We observed sedentarism rates at sea level, 1,600, and 2,500 m of 68, 75, and 68%, respectively, which is consistent with previously described levels of overweight and obese people. To estimate EET, we considered 1.55 as a cut off factor for physical activity at all three altitude levels (**Table 3**) and observed that subjects with an average EET above 110% at sea level, 1,600, and 2,500 m was 67, 58.8, and 45%, respectively. EET above 110% would tend to promote weight gain, therefore, the higher the energy balance percentage, the greater the risk of developing overweight or obesity.

*Cardiorespiratory Variables and hs*CRP Plasma Concentration at Sea Level, 1,600, and 2,500 m

We performed cardiorespiratory evaluations at each altitude and observed a progressive decline in arterial oxygenation with increasing altitude (**Table 4**). Conversely, cardiovascular variables such as systolic and diastolic blood pressure and HR increased with altitude (**Table 4**). Additionally, we observed that *hs*-CRP levels in plasma significantly increased at 2,500 m, compared to sea level and 1,600 m (**Figure 1**), and we observed a significant increase in the percentage of subjects with values over 3 mg/L (which indicates an increased risk of cardiovascular disease, see **Table 1**); 33.3% at 2,500 m compared with 9.6 and 8.5% at sea level and 1,600 m, respectively. **Supplementary Table** shows the correlation between *hs*CRP vs. anthropometrical and cardiometabolic variables, where we observed a positive and significant correlation with SAP and a negative and significant correlation with VO_2 max (expressed mlO2/min/kg and mlO2/min/kg LBM) at 2,500 m. Additionally, a positive and significant correlation was observed with BF, LBM, and WC and a negative and significant correlation was observed with VO_2 max (mlO2/min/kg).

Maximum Oxygen Consumption (VO_2 max)

Resting and maximum values of cardiorespiratory and metabolic parameters at sea level, 1,600, and 2,500 m are shown in **Table 5**.

We observed a significant difference between resting and maximum exercise in VO_2, SpO2, HR, VE, and RER ($p < 0.05$). Additionally, when we compared cardiorespiratory and metabolic responses obtained at rest at sea level and high altitude, we observed a significant decline in oxygen saturation at 1,600 and 2,500 m ($p < 0.05$) and a significant increase in HR at 2,500 m ($p < 0.05$). No difference was observed in VO_2, VE, and RER ($p > 0.05$). However, when we compared cardiorespiratory and metabolic responses obtained at maximum exercise, we observed a progressive decline in maximum oxygen consumption (expressed mlO2/min/kg) at 2,500 m ($p < 0.05$). We observed a similar pattern when comparing maximum oxygen consumption and LBM. These low VO_2 max levels were obtained at the same intensity (Watts), RER, VE, and HR at sea level, 1,600, and 2,500 m. However, a gradual decrease in oxygen saturation was observed during maximum exercise at 1,600 and 2,500 m ($p < 0.05$).

DISCUSSION

Our results show that miners who work at moderate altitudes must maintain a similar workload, VE, and HR with decreased oxygen saturation and VO_2 max, and therefore must endure a greater cardiovascular challenge than those at sea level. Additionally, we observed an increased prevalence of sedentarism and overweight and obesity in all groups, and moderate altitude resulted in increased *hs*-CRP levels.

Corporal Composition and Nutrition

Our study shows that the average anthropometric values (BMI, BF, MF, WC) in the miners were higher than the national average according to the 2010 National Health Survey (Ministry of Health Chile, n.d.). Similar results were described in a Chilean miner population with CIHH exposure (Pedreros et al., 2018) who presented higher obesity and overweight rates. Additionally, increased blood pressure, cholesterol, and glucose levels were observed in workers of the mining industry exposed to intermittent high-altitude hypoxia at 3,700–4,000 m in the Kyrgyz Republic (Esenamanova et al., 2014). However, in a prospective study performed in Chilean miners without previous

TABLE 3 | Energetic balance in population that work at several altitudes.

	Altitude (m)		
	Sea level	**1,600**	**2,500**
Intake (Kcal/day)	2,305 + 232	2,341 + 512	2,940 + 409*†
Lost (Kcal/day)	2,102 + 154	2,229 + 192*	2,507 + 231*
Energetic balance	110	105	117
Energetic balance (<110%)	65.9	51.9	58.3

*Mean ± SD vs. sea level.
†Mean ± SD vs. 1,600 m ($p < 0.05$).

TABLE 4 | Basal cardiorespiratory evaluated in population that work at several altitudes.

	Altitude (m)		
	Sea level	**1,600**	**2,500**
Oxygen saturation (%)	98.5 ± 0.9	94.0 ± 1.1*	92.8 ± 1.3*†
Heart rate (bpm)	70.4 ± 8	75.1 ± 11.5	78.6 ± 10.9*
Systolic arterial pressure (mmHg)	127.8 ± 10.6	131.7 ± 11.3*	125.5 ± 8.8
Dyastolic arterial pressure (mmHg)	77.2 ± 9.3	86.5 ± 8.2*	83.3 ± 7.9

*Mean ± SD vs. sea level.
†Mean ± SD vs. 1,600 m ($p < 0.05$).

exposure to high altitude (3,800–4,600 m), it was shown that BW and body composition did not change significantly after 31-month (Richalet et al., 2002). The anthropometric information we collected in the present study is contradictory to that indicated in the literature. In the literature, it has been described that exposure to altitude under an acute exposure model leads to weight loss, due to a negative energy balance mainly caused by an increased basal metabolic rate and suppression of appetite (Lippl et al., 2010). Some studies have even shown that staying in a hypobaric hypoxic environment could even be used as a treatment for obesity due to the reasons noted above (Lippl et al., 2010; Palmer and Clegg, 2014; Karl et al., 2018). However, regarding our result, we do not observe that this positive effect translates into a reduction in overweight and obesity in our population. We believe that our results are due to lifestyles where energy intake exceeds expenditure (Vearrier and Greenberg, 2011), which could be partially explained by cultural aspects, such as the mining organizational structure, workers union pressure, and mining worker food-culture. A study published on miners with cardiovascular risk factors showed that the daily energy intake that a worker consumes could reach 6,378 Kcal/day, and was largely made up of energy-dense foods rich in simple sugars, sodium, cholesterol, saturated fatty acids, and with a low fiber content (Padilla, 1999). However, another study classified miner's physical activities as low in administrative workers (estimation of 2.2–2.4 Kcal/day), moderate in truck operators (estimation of 2.4–2.6 Kcal/day), and active in mechanical workers (estimated 2.8–3.0 Kcal/day; Caichac et al., 2013). In our study, we considered the population represented by truck operators to have a physical activity rate as light or sedentary.

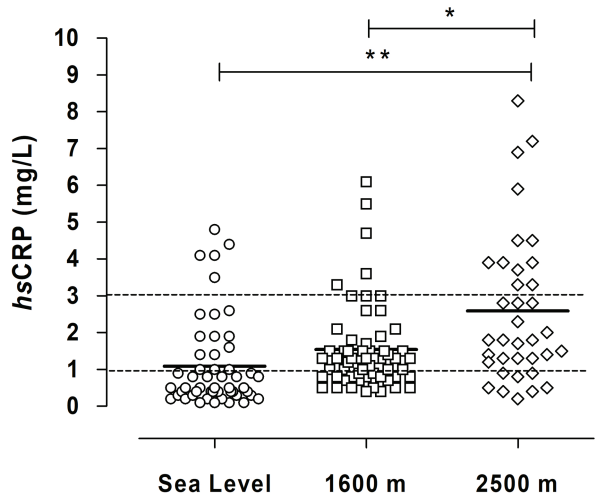

FIGURE 1 | Plasma concentration of *hs*CRP in miners exposed to CIHH at several altitudes. The horizontal thick line represents a mean value of *hs*CRP at each altitude. The horizontal dotted line represents a cut-off of cardiovascular risk (<1 mg/L, low; >1 and <3, moderate; and >3 high). Asterisks represent a significant difference between sea level and 2,500 m ($p < 0.05$) and double asterisks represent a significant difference between 1,600 and 2,500 m ($p < 0.05$).

TABLE 5 | Resume of cardiorespiratory variables evaluated at maximum exercise that work at several altitudes.

	Altitude (m)		
	Sea level	**1,600**	**2,500**
Intensity (Watts)			
Rest	0	0	0
Maximum	174 ± 33	198 ± 25	173 ± 27
VO_2 (mlO_2/kg/min)			
Rest	3.6 ± 0.7	3.5 ± 0.9	3.1 ± 0.8
Maximum	25.9 ± 5.8†	22.9 ± 4.8†	19.2 ± 5.3*†
VO_2 (mlO_2/kg (BLM)/min)			
Rest	5.0 ± 1.0	4.8 ± 1.2	4.2 ± 1.1
Maximum	36.1 ± 6.2†	31.2 ± 5.1†	26.6 ± 6.2*†
RER			
Rest	0.84 ± 0.09	0.90 ± 0.10	0.86 ± 0.07
Maximum	1.20 ± 0.19†	1.27 ± 0.15†	1.23 ± 0.17†
VE (L/min)			
Rest	8.9 ± 2.4	9.4 ± 3.1	9.6 ± 2.2
Maximum	65.6 ± 17.2†	79.3 ± 18.8†	69.3 ± 18.4†
Oxygen saturation (SpO2, %)			
Rest	97.5 ± 0.5	94.5 ± 1.8*	92.8 ± 1.2*‡
Maximum	93.2 ± 1.1†	90.2 ± 1.7*†	88.3 ± 1.9*†
HR (bpm)			
Rest	71 ± 11	76 ± 10	80 ± 12*
Maximum	151 ± 16†	164 ± 14†	154 ± 16†

**Mean ± SD vs. sea level ($p < 0.05$).*
†Mean ± SD vs. rest ($p < 0.05$).
‡Mean ± SD vs. 1,600 m.
Heart rate (HR), Pulse oximetry (SpO2), and Ventilation (VE).

Cardiovascular Effects

High altitude is associated with increased sympathetic tone and may result in elevated blood pressure (Richalet et al., 1992; Naeije, 2010). In a previous study, the authors measured arterial blood pressure for 24 h in miners at sea level and high altitude (3,800 m) and found that the mean arterial pressure at high altitude was higher than at sea level, supporting the notion that high altitude leads to increased sympathetic tone (Richalet et al., 2002). Additionally, the authors found a reduction in mean arterial pressure after 31 months of exposure to CIHH. A similar response was described in the miner's population exposed to CIHH at 4,000 m (Kyrgyzstan; Vinnikov et al., 2016). In contrast, in another miner population exposed to CIHH at 3,700–4,000 m, high overweight and obesity rates are prevalent and associated with increased blood pressure (Esenamanova et al., 2014). However, in our study, we did not observe any changes and /or presence of subjects with elevated arterial pressure in the population exposed to sea level compared to 1,600 or 2,500 m.

We observed a significant increase in *hs*CRP plasma concentration at 1,600 and 2,500 m in miners exposed to CIHH. It was previously shown that non-specific inflammation could be induced by hypoxia and contribute to high altitude-associated diseases. Three studies have found increased levels of IL-6 and CRP at high altitude compared to baseline levels during both passive or active ascent of Capanna Regina Margherita (4,559 m). IL-6 peaked on the second day and

declined to baseline during the following 3 days. Additionally, CRP levels increased on day 3 and remained elevated before descending (Hartmann et al., 2000; Bailey et al., 2004). A study performed on subjects without previous prolonged exposure to high altitude showed that these people presented increased plasma *hs*CRP levels when they stayed at 4,000 m for 3 months (Hu et al., 2016). In these studies, the authors proposed that hypoxia at high altitudes could be considered a new inflammatory stimulus below the range expected for inflammatory diseases, such as acute mountain sickness or HAPE. Early studies describe a high increase in IL-6 and CRP levels with HAPE in subjects at 4,559 m (Kleger et al., 1996). However, no miner exposed to CIHH at 4,500 m suffered from severe forms of mountain sickness (HAPE or HACE; Richalet et al., 2002) and an incidence of HAPE 0.49% and no coronary events were observed during the construction of the Qinghai-Tibet railroad (Wu et al., 2007). Also, in our study, we did not observe HAPE at 1,600 and 2,500 m. The higher *hs*CRP values in miners could also be explained by the high prevalence of overweight and obesity levels previously described: since obesity (excess adipose tissue) is characterized by a state of permanent mild inflammation with increased circulating levels of inflammatory markers such as *hs*CRP, IL-6, TNF∝, and others (more detail see Kayser and Verges, 2013). However, the high prevalence of overweight and obesity described in the present study is not related to altitude, suggesting that the observed increase is the result of altitude exposure. A study performed in Puno-Perú (3,825 m) show elevated values of *hs*CRP (>3 mg/L) in individuals with lower values of oxygen saturation (Miele et al., 2016). Also, lower resting daytime oxygen saturation may serve as a marker of increased cardiovascular risk at high altitudes (Grundy et al., 2004).

Maximal Oxygen Consumption at High Altitude

Many studies have reported the fall in VO_2 max at high altitudes (see West, 2006). A study performed by Cerretelli (1980) reports that this fall is consistent with acute or chronic exposure and Kayser (2005) reported a VO_2 maximum decrease by 1% for every additional 100 m elevation over 1,500 m. Therefore, VO_2 max could decrease by 1–2% at 1,600 m and a reduction of 10% in the VO_2 max at 2,500. However, our results showed a reduction of 11.6% of VO_2 max at 1,600 m and 25.9% at 2,500 m, a difference of nearly 10% at 1,600 m and 15.9% at 2,500 m. We previously described a 29% reduction in VO_2 max in miners exposed CIHH at 3,800 m. This reduction was similar to that described by other authors associated with a decreased intensity (Sutton et al., 1988). However, in our previous studies, this fall in VO_2 max does not correlate with a fall in intensity and was interpreted as an increase in work efficiency at 3,800 m (Moraga et al., 2018, 2019) due to a major effort in the respiratory muscle work (see discussion, Moraga et al., 2019). In contrast, we reported a reduction of 25.9% in the VO_2 max at 2,500 m where the intensity of exercise, VE, and HR was lower than that described at 3,800 m (Moraga et al., 2019). A possible explanation for the lower aerobic capacity in our study population could be a worse physical condition, since over 70% of subjects were sedentary, overweight, and aged. This worse physical condition could be corroborated by values of maximal effort (extenuating) at the lower value of 200 Watts at 1,600 and 2,500 m and lower HR values. Similarly, a decreased aerobic capacity in miners was previously reported at an intensity of 175 W, representing a decreased maximal intensity of ~15% after 31 months of CIHH exposure in subjects evaluated at sea level (Richalet et al., 2002). Reduced HR was also explained by the downregulation of β-adrenergic receptors, upregulation of muscarinic receptors (Richalet et al., 1992), and a detraining effect of exposure to hypoxia and/or being excessively sedentary (Richalet et al., 2002). We considered that the lower VO_2 max values we observed in the present study could be due to a significant FM. However, when we calculated VO_2 max/LBM (kg), values of VO_2 max were enhanced but the percentage of change in VO_2 max at an altitude of 1,600 and 2,500 m compared to sea level was maintained (**Table 4**). This evidence suggests that the fall in VO_2 max is due to a lower aerobic capacity as a result of sedentarism, rather than an increase in the fatty mass in our population.

LIMITATIONS

We recognize a series of limitations in our assessment of miners who work at sea level or a geographic altitude of 1,600 and 2,500 m. For instance, it was impossible to evaluate the effect of exposure to high altitude on cardiorespiratory variables during the VO_2 max test because unions opposed the use of rest time (descending) for evaluation. Our study did not evaluate other metabolic and cardiovascular biomarkers such as oxidative stress and/or antioxidant mechanisms. Gender equity studies in this population are very difficult since women workers at altitude (low, moderate, and high) are very low or anecdotal. However, the population of women that work at altitude is associated primarily with functional services (health, feeding, cleaning, and others). In future studies, we will consider monitoring arterial blood pressure for 24 h and evaluate other markers of endothelium dysfunction (NO, ADMA, Homocysteine) to obtain a better understanding of the mechanisms underlying the high-altitude physiological adjustments in this population of shift workers.

Conclusion

We found that work capacity, HR, and VE with a decreased VO_2 max is maintained at moderate altitude, suggesting that work efficiency is maintained. Higher prevalence of overweight-obesity, BF% and WC, and sedentarism in all miner populations are evidence of cardiometabolic risk that is not related to altitude. However, increased *hs*CRP levels were associated with altitudes of 1,600 and 2,500 m.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Facultad de Medicina, Universidad Católica del Norte, Chile. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FM conceived and designed the study. RC-J and AP-L supervised the overall study. RC-J performed the statistical analysis. DM, RC-J, and AP-L contributed to sample and data collections. All authors drafted the report and contributed to the interpretation of the results, critical revision of the manuscript, and approval of the final manuscript. FM is the guarantor.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.647976/full#supplementary-material

REFERENCES

Ainsworth, B. E., Haskell, W. L., Whitt, M. C., Irwin, M. L., Swartz, A. M., Strath, S. J., et al. (2000). Compendium of physical activities: an update of activity codes and MET intensities. *Med. Sci. Sports Exerc.* 32, S498–S516. doi: 10.1097/00005768-200009001-00009

Bailey, D. M., Kleger, G. R., Holzgraefe, M., Ballmer, P. E., and Bärtsch, P. (2004). Pathophysiological significance of peroxidative stress, neuronal damage, and membrane permeability in acute mountain sickness. *J. Appl. Physiol.* 96, 1459–1463. doi: 10.1152/japplphysiol.00704.2003

Bray, G. A. (1998). What is the ideal body weight? *J. Nutr. Biochem.* 9, 489–492. doi: 10.1016/S0955-2863(98)00007-2

Caichac, A., Mediano, F., Blanco, G., Lera, L., Yañez, C., Vio, F., et al. (2013). Food and nutrition intervention for miners with cardiovascular risk factors, based on formative research. *Rev. Chil. Nutr.* 40, 336–342. doi: 10.4067/S0717-75182013000400002

Calbet, J. A. L., and Lundby, C. (2009). Air to muscle O2 delivery during exercise at altitude. *High Alt. Med. Biol.* 10, 123–134. doi: 10.1089/haam.2008.1099

Cerretelli, P. (1980). "Gas exchange at high altitude" in *Pulmonary gas exchange. Vol. II*. ed. J. B. West (New York: Academic Press), 97–147.

Durnin, J., and Womersley, J. (1974). Body fat assessment from total body density and its estimation from skindfold thickness: measurements on 481 men and women aged from 16 to 72 years. *Br. J. Nutr.* 32, 77–97. doi: 10.1079/bjn19740060

Esenamanova, M. K., Kochkorova, F. A., Tsivinskaya, T. A., Vinnikov, D., and Aikimbaev, K. (2014). Chronic intermittent high altitude exposure, occupation, and body mass index in workers of mining industry. *High Alt. Med. Biol.* 15, 412–417. doi: 10.1089/ham.2013.1150

FAO/WHO/UNU (2004). Human Energy Requirements. Joint FAO/WHO/UNU Expert Consultation. FAO Food and Nutrition Technical Report Series no. 1. Rome.

Grundy, S. M., Hansen, B., Smith, S. C. Jr., Cleeman, J. I., Kahn, R. A. and American Heart Association, National Heart, Lung, and Blood Institute, American Diabetes Association (2004). Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung, and Blood Institute/ American Diabetes Association conference on scientific issues related to management. *Circulation* 109, 433–438.

Hartmann, G., Tschöp, M., Fischer, R., Bidlingmaier, C., Riepl, R., Tschöp, K., et al. (2000). High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. *Cytokine* 12, 246–252. doi: 10.1006/cyto.1999.0533

Hu, S. L., Xiong, W., Dai, Z. Q., Zhao, H. L., and Feng, H. (2016). Cognitive changes during prolonged stay at high altitude and its correlation with C-reactive protein. *PLoS One* 11:e0146290. doi: 10.1371/journal.pone.0146290

Jensen, M. D., Ryan, D. H., Apovian, C. M., Ard, J. D., Comuzzie, A. G., Donato, K. A., et al. (2013). AHA/ACC/ TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association task force on practice guidelines and the Obesity Society. *Circulation* 129, S102–S138. doi: 10.1161/01.cir.0000437739.71477.ee

Karl, J. P., Cole, R. E., Berryman, C. E., Finlayson, G., Radcliffe, P. N., Kominsky, M. T., et al. (2018). Appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at high altitude, but are not affected by protein intake. *High Alt. Med. Biol.* 19, 156–169. doi: 10.1089/ham.2017.0155

Kayser, B. (2005). Why is endurance performance decreased at high altitude? *Schweiz Zeitschr Sportmed Sporttraum* 53, 54–60.

Kayser, B., and Verges, S. (2013). Hypoxia, energy balance and obesity: from pathophysiological mechanisms to new treatment strategies. *Obes. Rev.* 14, 579–592. doi: 10.1111/obr.12034

Kleger, G. R., Bärtsch, P., Vock, P., Heilig, B., Roberts, L., and Ballmer, P. E. (1996). Evidence against an increase in capillary permeability in subjects exposed to high altitude. *J. Appl. Physiol.* 81, 1917–1923. doi: 10.1152/jappl.1996.81.5.1917

Libby, P., Ridker, P. M., and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation* 105, 1135–1143. doi: 10.1161/hc0902.104353

Lippl, F. J., Neubauer, S., Schipfer, S., Lichter, N., Tufman, A., Otto, B., et al. (2010). Hypobaric hypoxia causes body weight reduction in obese subjects. *Obesity* 18, 675–681. doi: 10.1038/oby.2009.509

Macek, P., Biskup, M., Terek-Derszniak, M., Krol, H., Smok-Kalwat, J., Gozdz, S., et al. (2020). Optimal cut-off values for anthropometric measures of obesity in screening for cardiometabolic disorders in adults. *Sci. Rep.* 10:11253. doi: 10.1038/s41598-020-68265-y

Miele, C. H., Schwartz, A. R., Gilman, R. H., Pham, L., Wise, R. A., Davila-Roman, V. G., et al. (2016). Increased cardiometabolic risk and worsening hypoxemia at high altitude. *High Alt. Med. Biol.* 17, 93–100. doi: 10.1089/ham.2015.0084

Ministry of Health Chile (n.d.). Available at: https://www.minsal.cl/portal/url/item/bcb03d7bc28b64dfe040010165012d23.pdf

Moraga, F. A., Osorio, J., Calderón-Jofré, R., and Pedreros, A. (2018). Hemoconcentration during maximum exercise in miners with chronic intermittent exposure to hypobaric hypoxia (3800 m). *High Alt. Med. Biol.* 19, 15–20. doi: 10.1089/ham.2017.0011

Moraga, F. A., Osorio, J., Jiménez, D., Calderón-Jofré, R., and Moraga, D. (2019). Aerobic capacity, lactate concentration, and work assessment during maximum exercise at sea level and high altitude in miners exposed to chronic intermittent hypobaric hypoxia (3,800 m). *Front. Physiol.* 10:1149. doi: 10.3389/fphys.2019.01149

Naeije, R. (2010). Physiological adaptation of the cardiovascular system to high altitude. *Prog. Cardiovasc. Dis.* 52, 456–466. doi: 10.1016/j.pcad.2010.03.004

Olivares, A., Bustos, N., Weisstaub, G., and Lera, L. (2007). *Estado nutricional, estudios de laboratorio, encuesta alimentaria, actividad física y consumo de tabaco en trabajadores de empresas afiliadas al INP*. Santiago: Informe Técnico INTA, Universidad de Chile.

Padilla, M. (1999). "Regulación del metabolismo de lípidos, proteínas, hidratos de carbono y otros nutrientes en trabajadores en altitud" in *Nutrición, fisiología y ejercicio en altitud*. Santiago, Chile: Asociación Chilena de Seguridad and Universidad de Antofagasta, 67–78.

Palmer, B. F., and Clegg, D. C. (2014). Ascent to altitude as a weight loss method: the good and bad of hypoxia inducible factor activation. *Obesity* 22, 311–317. doi: 10.1002/oby.20499

Pedreros, A., Calderón, R., and Moraga, F. A. (2018). Estado nutricional, composición corporal e indicadores antropométricos de trabajadores mineros expuestos a hipoxia hipobárica crónica e intermitente a una altitud moderada (0-2500 msnm). *Rev. Chil. Nutr.* 45, 199–204. doi: 10.4067/S0717-75182018000400199

Pepys, M. B., and Hirschfield, G. M. (2003). C-reactive protein: a critical update. *J. Clin. Invest.* 111, 1805–1812. doi: 10.1172/JCI18921

Prommer, N., Heinicke, K., Viola, T., Cajigal, J., Behn, C., and Schmidt, W. F. J. (2007). Long term intermittent hypoxia increases O2 transport capacity but not VO_2 max. *High Alt. Med. Biol.* 8, 225–235. doi: 10.1089/ham.2007.8309

Richalet, J. P., Kacimi, R., and Antezana, A. M. (1992). The control of cardiac chronotropic function in hypobaric hypoxia. *Int. J. Sports Med.* 13, S22–S24. doi: 10.1055/s-2007-1024582

Richalet, J. P., Vargas Donoso, M., Jiménez, D., Antezana, A. M., Hudson, C., Cortés, G., et al. (2002). Chilean miners commuting from sea level to 4500 m: a prospective study. *High Alt. Med. Biol.* 3, 159–166. doi: 10.1089/15270290260131894

Shah, S. M., Nanan, D., Rahbar, M. H., Rahim, M., and Nowshad, G. (2004). Assessing obesity and overweight in a high mountain Pakistani population. *Tropical Med. Int. Health* 9, 526–532. doi: 10.1111/j.1365-3156.2004.01220.x

Sutton, J. R., Reeves, J. T., Wagner, P. D., Groves, B. N., Cymerman, A., Malconian, M. K., et al. (1988). Operation Everest II: oxygen transport during exercise at extreme simulated altitude. *J. Appl. Physiol.* 64, 1309–1321. doi: 10.1152/jappl.1988.64.4.1309

Vearrier, D., and Greenberg, M. (2011). Occupational health of miners al altitude: adverse health effects, toxic exposure, pre-placement screening, acclimatization, and worker surveillance. *Clin. Toxicol.* 49, 629–640. doi: 10.3109/15563650.2011.607169

Vinnikov, D., Brimkulov, N., and Krasotski, V. (2016). Chronic intermittent hypoxia and blood pressure: is there risk for hypertension in healthy individuals? *High Alt. Med. Biol.* 17, 5–10. doi: 10.1089/ham.2015.0067

West, J. B. (2006). Human responses to extreme altitude. *Integr. Comp. Biol.* 46, 25–34. doi: 10.1093/icb/icj005

Westerterp, K. R. (2017). Control of energy expenditure in humans. *Eur. J. Clin. Nutr.* 71, 340–344. doi: 10.1038/ejcn.2016.237

Wu, T. Y., Ding, S. Q., Lui, J. L., Yu, M. T., Jia, J. H., Chai, Z. C., et al. (2007). Who should not go high: chronic disease and work at altitude during construction of the Qinghai-Tibet railroad. *High Alt. Med. Biol.* 8, 88–107. doi: 10.1089/ham.2007.1015

Yudkin, J. S., Stehouwer, C. D., Emeis, J. J., and Coppack, S. W. (1999). C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler. Thromb. Vasc. Biol.* 19, 972–978. doi: 10.1161/01.atv.19.4.972

Zacarías, I., Barrios, L., González, C., Loef, T., and Vera, G. (2018). *Food composition table*. Santiago: Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, 219.

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A Prospective Evaluation of the Acute Effects of High Altitude on Cognitive and Physiological Functions in Lowlanders

Marika Falla[1,2], Costanza Papagno[2,3], Tomas Dal Cappello[1], Anna Vögele[1], Katharina Hüfner[4], Jenny Kim[5], Elisabeth M. Weiss[6], Bernhard Weber[7], Martin Palma[1], Simona Mrakic-Sposta[8], Hermann Brugger[1] and Giacomo Strapazzon[1]*

[1]Institute of Mountain Emergency Medicine, Eurac Research, Bolzano, Italy, [2]Center for Mind/Brain Sciences CIMeC, University of Trento, Rovereto, Italy, [3]Department of Psychology, University of Milano-Bicocca, Milan, Italy, [4]Department of Psychiatry, Psychotherapy and Psychosomatics, Division of Psychiatry II, Medical University of Innsbruck, Innsbruck, Austria, [5]Institute for Exercise and Environmental Medicine, Texas Health Presbyterian Hospital, Dallas, TX, United States, [6]Department of Psychology, University of Innsbruck, Innsbruck, Austria, [7]Department of Psychology, University of Graz, Graz, Austria, [8]Institute of Clinical Physiology, National Research Council (CNR), Milan, Italy

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Charles Limoli, University of California, Irvine, United States

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***Correspondence:**
Marika Falla
marika.falla@unitn.it

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Cognitive function impairment due to high altitude exposure has been reported with some contradictory results regarding the possible selective cognitive domain involvement. We prospectively evaluated in 36 lowlanders, exposed for 3 consecutive days to an altitude of 3,269 m, specific cognitive abilities (attention, processing speed, and decision-making) required to safely explore the mountains, as well as to work at altitude. We simultaneously monitored the physiological parameters. Our study provides evidence of a reduced processing speed in lowlanders when exposed to altitude in the first 24 h. There was a fairly quick recovery since this impairment was no more detectable after 36 h of exposure. There were no clinically relevant effects on decision-making, while psychomotor vigilance was unaffected at altitude except for individuals with poor sleep. Significant changes were seen in physiological parameters (increased heart rate and reduced peripheral oxygen saturation). Our results may have practical implications, suggesting that individuals should practice prudence with higher ascent when performing risky activities in the first 24–36 h, even at altitudes below 3,500 m, due to an impairment of the cognitive performance that could worsen and lead to accidents.

Keywords: altitude, cognitive functions, speed-processing, decision-making, attention

INTRODUCTION

There is increasing mountain attendance related to different recreational risky activities (e.g., mountaineering, skiing, and climbing), as well as for occupational purposes (e.g., mining, astrophysics) with consequently increasing accidents (Monasterio, 2005). Preserved cognitive functions, such as executive function, attention, and memory, are essential during such activities since a reduced efficiency of those abilities can provoke injury or even death in such environments. Severe acute hypoxia or anoxia was found to be related to impairment in executive function, attention, and memory (van Alem et al., 2004). Ascent to high altitude (HA) precipitates a drop in the barometric pressure and the atmospheric partial pressure of oxygen (O_2), a condition termed as hypobaric

hypoxia (HH; Taylor, 2011). The reduction of oxygen availability induces physiological changes to maintain adequate oxygen delivery, especially into the brain. The acute exposure to HH induces increased ventilation, an autoregulatory increase in cerebral blood flow and an increased oxygen extraction at the tissue/cell level. Despite these changes, a reduction in the total amount of oxygen available persists, producing a decrease in cognitive performance and different HA illnesses, especially if ascent occurs too rapidly with no acclimatization. Hornbein et al. (1989) found a slight decline in verbal and visual long-term memory and increased errors in the aphasia screening test in mountaineers exposed to altitude between 5,488 and 8,848 m.

Current results are controversial, and it is not yet clear whether cognitive abilities are selectively impaired or there is a general cognitive impairment. McMorris et al. (2017) performed a systematic review and meta-analysis on the acute effect of hypoxia on cognition. They included 18 studies, and they observed that hypoxia (both normobaric and hypobaric; arterial partial pressure of oxygen range between 35 and 89 mmHg) exerts a negative effect on cognition on both tasks investigating central executive (working memory set-shifting, updating, monitoring, inhibition, and planning) and non-executive (perception, attention, and short-term memory) functions. In a more recent review and meta-analysis, the effect of hypoxia on cognition was further confirmed, but the authors observed a selective effect: information processing seems to be enhanced (mainly in Females), whereas attention, executive function and memory impaired (Jung et al., 2020). In the 18 included studies the fraction of inspired oxygen ranged from 10 to 18%. Differences in altitude-exposure speed, duration and profile, the way of ascent, study population, cognitive tests employed, and test administration times at altitude (Li et al., 2000; Pavlicek et al., 2005; De Bels et al., 2019; Loprinzi et al., 2019) can explain discrepancies and hinder the drawing of conclusions regarding the effects of altitude on the cognition of recreationists.

Our aim was to prospectively evaluate specific cognitive functions (attention, speed processing, and decision-making) required to safely explore the mountains, as well as to work at altitude. We wanted to assess whether acute HH exposure impairs all these cognitive functions or produces selective effects on specific ones in lowlanders exposed for 3 consecutive days to an altitude of 3,269 m. At such altitude several mountain huts, winter resorts, and different occupational infrastructures are located worldwide. We also examined the correlation between cognitive performances and physiological parameters evaluated at the same timeline.

MATERIALS AND METHODS

Participants

Participants were recruited among medical doctors or nurses participating in a mountain medicine course held in the Northern Italian Alps (Ortles-Cevedale group) at Casati hut (3,269 m). All the participants had experience in trekking. Inclusion criteria were male and female participants with an age between 18 and 60 years. Exclusion criteria was age outside that range. The study and the informed consent procedure were approved by the Institutional Review Board of Bolzano (Protocol Number 812020-BZ). The study was conducted according to the Declaration of Helsinki and reported in accordance with the START Data Reporting Guidelines for Clinical High Altitude Research (Brodmann et al., 2018).

Study Protocol

A longitudinal study design was performed within 3 summer days. Each participant underwent neurocognitive testing on a dedicated personal computer (PC) four times plus a familiarization session, along with the completion of several questionnaires and physiological parameters' assessment individually and quietly (see **Figure 1**). All participants were asked to reach the baseline testing site staggered in groups of four individuals and at different arrival times (between 8:00 and 12:00 AM). They were initially studied in the morning for the baseline test near the trekking route (Ponte di Legno, 1,258 m; session 1, day 1, D1 S1). Participants then in groups of four drove to the parking location (2,178 m) and trekked to the Casati hut on foot along the same route (around 3:30 h). Participants were further assessed three times at altitude (3,269 m) upon arrival (session 2, day 1, D1 S2; between 6:00 and 10:00 PM), and early in the morning (between 6:00 and 8:00 AM) on the next 2 days (session 3, day 2, D2 S3, and session 4, day 3, D3 S4; see **Figure 1**). Before each session day (at least 2 h), participants were asked to avoid caffeine, tea, or alcohol intake. During day 2, all participants attended the mountain medicine course with minimal physical effort.

Measures

Demographical data (age, gender, education, height, weight, altitude of residency, pregnancy, and smoking), physical activity, oral medication, or any disease (above all any neurological or psychiatric disease) were recorded. Information on staying at altitude in the 3 previous days/nights, trip >2,500 m during the last 3 months, past altitude-illness events were recorded. Physiological parameters, such as heart rate (HR) and peripheral oxygen saturation (SpO_2), were measured in all sessions, after resting period, and in a warm and comfortable environment.

Questionnaires on Mood, Sleep, Stress, Resilience, and Mountain Sickness

All participants completed multiple questionnaires. The administration timeline (session 1–4) of the different tests is shown in **Figure 1**. Anxiety and depression were evaluated using the hospital anxiety and depression scale (HADS; Zigmond and Snaith, 1983) and the State Trait Anxiety Inventory (STAI-Y1-*state* and -Y2-*trait*; Spielberger et al., 1983). *State* anxiety is a transient reaction to adverse events in a specific moment, and the *trait* anxiety is a more stable personality characteristic. Sleep quality was evaluated at baseline (session 1) using the Pittsburgh Sleep Quality Index (PSQI; Buysse et al., 1989), a questionnaire that assesses sleep quality and quantity over a month-long period. Additionally, at sessions 2, 3, and 4 the Insomnia Severity Index (ISI; Morin et al., 2011), a self-report measure that assesses

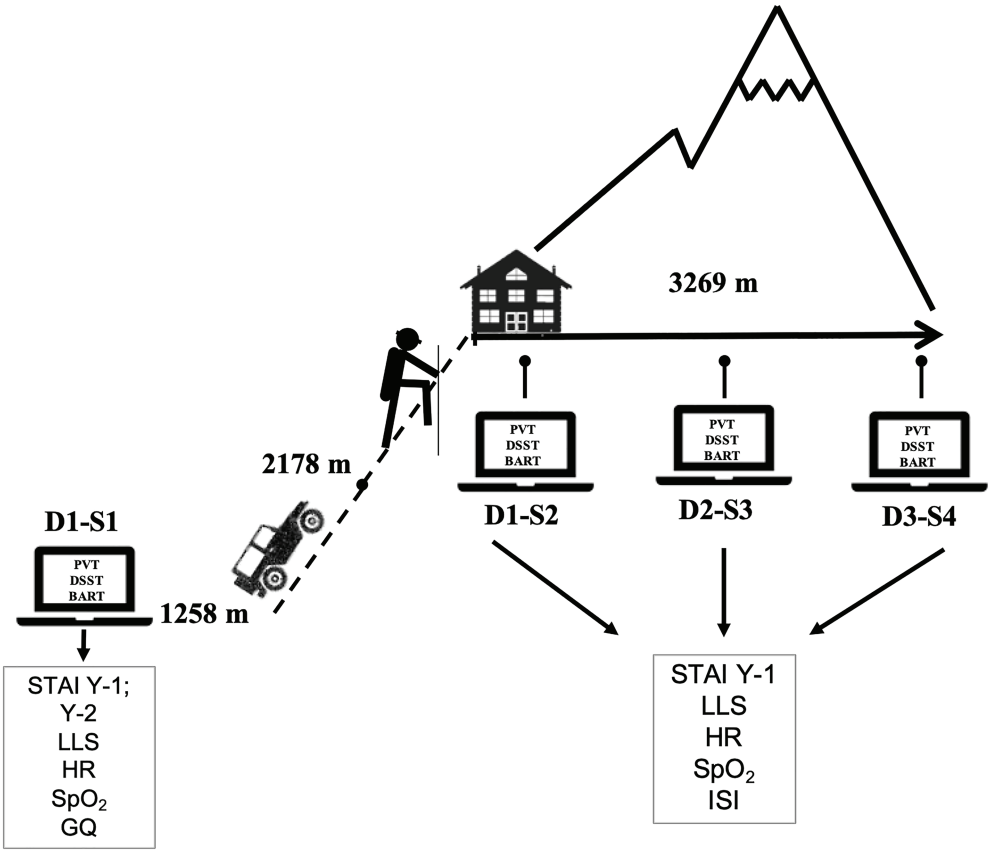

FIGURE 1 | Timeline of cognitive test, questionnaires administration, and physiological-parameter recording. BART, Balloon Analogue Risk Task; D, day; DSST, Digit Symbol Substitution Test; GQ, general questionnaire; HR, heart rate; ISI, Insomnia Severity Index; LLS, Lake Louise Score; PVT, Psychomotor Vigilance Test; S, session; SpO_2, peripheral oxygen saturation; STAI, State and Trait Anxiety Inventory (Y1-*state* and Y2-*trait*).

participants' perceptions of their insomnia over the previous night was used. Stress was evaluated using the 10-item version of the Perceived Stress Scale (PSS-10; Cohen and Williamson, 1988), and resilience was investigated using the Wagnild and Young's scale (RS – 14; Wagnild and Young, 1993; Wagnild, 2009). Symptoms of acute mountain sickness (AMS) were evaluated using the Lake Louise Score (LLS; Roach et al., 2018).

Cognitive Tests

Three different cognitive tests on a portable personal computer were employed. The brief 3-min version of the Psychomotor Vigilance Test (PVT), similar to the one reported by Basner et al. (2011), evaluated sustained attention and response time (**Table 1**). The Balloon Analogue Risk Task (BART; Lejuez et al., 2002) evaluated the risky decision-making. The Digit Symbol Substitution Test (DSST) measures a range of cognitive performance including speed of processing and low-level visual search, and parallel forms were used to avoid practice effects (Wechsler, 2008). Randomized test sequences were also used across the four sessions. The cognitive stimuli were presented using PsychoPy (version 3.1.0),[1] and the software with the three cognitive tests was installed on four Eurac Research-issued laptops. To ensure that all laptops perform identically at various altitudes, laptop benchmark software (NovaBench) was run several times at different elevations.[2] The software achieved the same scores during all tests, leading to the conclusion that a difference in altitude has no impact on the laptop's performance.

Statistical Analysis

The Friedman test was used to compare LLS, STAI-Y1-*state*, HR, and SpO_2 during all four sessions and ISI during three sessions. Pairwise comparisons were analyzed by means of the Wilcoxon signed-rank test. The parameters of the cognitive tests (BART, DSST, and PVT) were analyzed by means of generalized estimating equations (GEE), considering the following factors: session (i.e., the time of exposure to altitude), gender, age (two groups, considering the median of 26 years as cut-off), cognitive tests sequence, whether LLS was ≥3 (i.e., in the presence of headache, it is considered diagnostic for AMS) either at sessions 2 or 3, ISI (two groups, 0–7 and ≥8), SpO_2 (two groups, <90 and ≥90%), and the interaction of session with gender. In the GEE, for BART mean earnings, BART mean pumps and PVT

[1]www.psychopy.org

[2]https://novabench.com/

TABLE 1 | Description of the cognitive tests.

Tests	Cognitive domain	Description	Outcome measures
Digit Symbol Substitution Test *(DSST)*	Processing speed and low-level visual search	At the bottom of the screen a fix legend is presented showing blue boxes containing numbers (1–9) and on the top pairing nonsense symbols. One of the nine symbols appears randomly on the center of the screen, and the participant must select the corresponding number as quickly as possible using the keyboard numbers in a row.	**Number of correct responses** **Number of incorrect responses**
Psychomotor Vigilance Test *(PVT)*	Sustained and vigilant attention	Simple reaction time (RT) to visual stimuli that occur at random intervals presented on a screen.	**Reaction time (RT)** [ms] **Lapses:** number of omission errors or RT ≥ 355 ms **False starts:** errors of commission defined as a response without a stimulus or a RT < 100 ms
Balloon Analogue Risk Taking *(BART)*	Risky decision making	Each participant has to inflate the balloon or to cash the current virtual value of the balloon. On every pump the balloon's size increases and can randomly explode. If a balloon explodes, the value of that balloon is lost but the previous total cashed value is unaffected. Goal is to achieve the greater reward balancing the possible loss.	**Total** amount of money **earned** **Total** count of **pumps** (only successful trials)

TABLE 2 | Demographical data (36 participants).

Features/Variables	Mean ± SD (range) or n (%)	Notes
Age, years	27.3 ± 4.1 (range 22–40)	
Females, n	18 (50%)	None pregnant
Education, years	18.9 ± 0.9 (range 16–21)	
Height, m	1.72 ± 0.09 (range 1.59–1.90)	
Weight, kg	63.8 ± 9.8 (range 48–85)	
Altitude of residence, m	238 ± 307 (range 0–1,200)	
Altitude of the 3 previous days/ nights, m	228 ± 301 (range 0–1,200)	
Sleep at >2,500 m during last 3 months, n	6 (16.7%)	
Daily trip >2,500 m during last 3 months:		
– number of trips, n	2.1 ± 4.0 (range 0–15)	
– number of participants, n	16 (44.4%)	
History of altitude illnesses:		
– past AMS, n	5 (13.9%)	
– past HACE, n	0 (0.0%)	
– past HAPE, n	0 (0.0%)	
Physical activity:		
– moderate level, n	18 (50.0%)	
– high level, n	18 (50.0%)	
Smoker, n	5 (13.9%)	
Neurological or psychiatric disease:		
– migraine, n	1 (2.8%)	
– anxiety, n	1 (2.8%)	
– depression, n	1 (2.8%)	
Medication, n	11 (30.6%)	7 on demand

AMS, acute mountain sickness; HACE, high-altitude cerebral edema; HAPE, high-altitude pulmonary edema; n, number of participants/times; SD, standard deviation.

TABLE 3 | Baseline questionnaires (36 participants).

Questionnaires	Mean ± SD (range) or n (%)
STAI-Y2-*trait*	34.5 ± 7.7 (range 22–58)
Participants with STAI-Y2 above threshold for age/gender	8 (22.2%)
PSQI (cut-off > 5)	4.4 ± 2.6 (range 1–12)
Participants with PSQI > 5	8 (22.2%)
HADS-A (cut-off ≥ 8)	4.2 ± 2.9 (range 0–10)
Participants with HADS-A ≥ 8	5 (13.9%)
HADS-D (cut-off ≥ 8)	1.5 ± 2.0 (range 0–7)
Participants with HADS-D ≥ 8	0 (0%)
PSS	11.2 ± 5.5 (range 3–25)
Participants with PSS low score (0–13)	24 (66.7%)
Participants with PSS moderate score (14–26)	12 (33.3%)
RS-14	82.5 ± 8.2 (range 65–97)

HADS, Hospital Anxiety Depression Scale; STAI-Y2-trait, State and Trait Anxiety Inventory; PSQI, Pittsburgh Sleep Quality Index; PSS, Perceived Stress Scale; RS-14, Resilience Scale 14 items.

mean reaction time, the normal distribution and identity as link function were specified, while for DSST, the number of correct and incorrect responses and PVT number of lapses and of false starts, the specified distribution and link function were the Poisson and the logarithm, respectively; for BART, total time of test execution the gamma distribution, and logarithm as link function were specified. The Holm-Bonferroni method was used to correct the p-values for multiple comparisons. SPSS version 25 statistical software (IBM Corp., Armonk, NY) was used. Tests were two-sided and $p < 0.05$ was considered as statistically significant. Values are reported as mean ± standard deviation and estimates of the GEE as mean (95% confidence interval, CI).

RESULTS

All 36 attendants of the mountain medicine course agreed to participate and were enrolled in the study. Demographical data are shown in **Table 2** (27.3 ± 4.1 year-old; 50% female; 18.9 ± 0.9 years of education). All were lowlanders and had slept at low altitude the three nights before testing; six (16.7%) slept higher

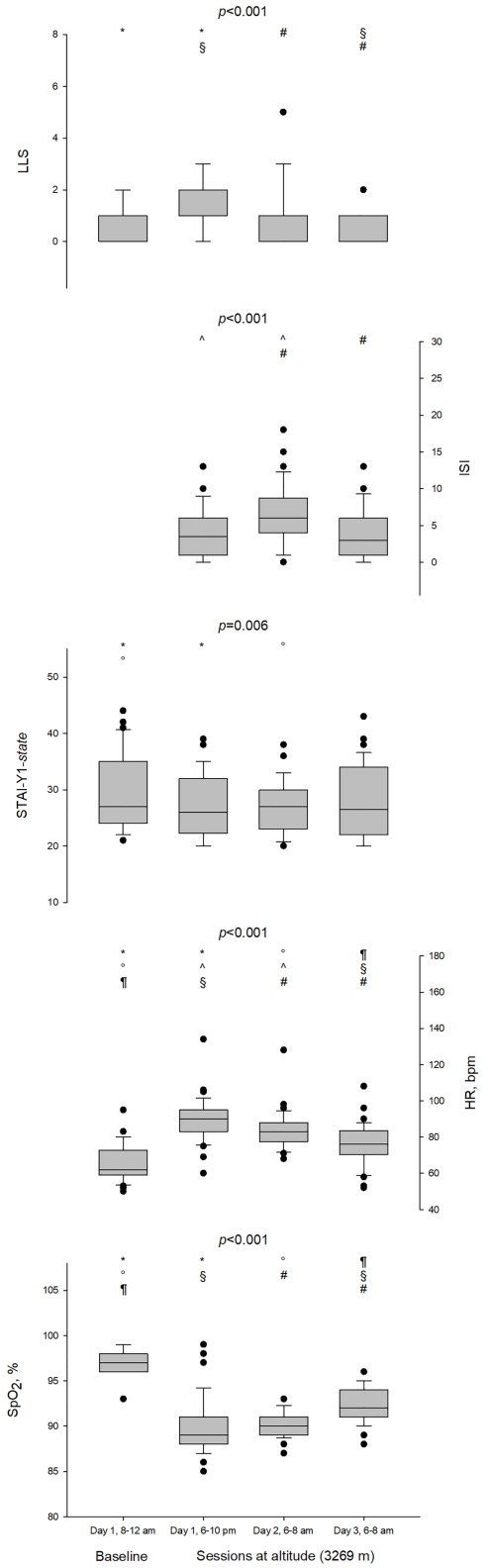

FIGURE 2 | Boxplots of Lake Louise Score (LLS), Insomnia Severity Index (ISI), State and Trait Anxiety Inventory (STAI-Y1-*state*), Heart rate (HR), and

(Continued)

FIGURE 2 | peripheral oxygen saturation (SpO_2) at baseline and sessions at altitude (3,269 m). Test performed was Friedman test. Pairwise comparisons were analyzed by means of Wilcoxon signed-rank test and the *p*-values were adjusted by means of Holm-Bonferroni correction. Statistically significant ($p < 0.05$) pairwise comparisons were denoted by the following symbols: *for session 1 (day 1, 8:00–12:00 AM) vs. session 2 (day 1, 6:00–10:00 PM), °for session 1 vs. session 3 (day 2, 6:00–8:00 AM), ¶for session 1 vs. session 4 (day 3, 6:00–8:00 AM), ^for session 2 vs. session 3, §for session 2 vs. session 4, and #for session 3 vs. session 4. bpm, beats per minute; •, outlier.

than 2,500 m, and 16 (44.4%) had made a daily trip above 2,500 m in the previous 3 months. While five participants had experienced AMS in the past, no one reported high altitude cerebral oedema (HACE) or high-altitude pulmonary oedema (HAPE). Only three participants suffered neurological (one migraine) or psychiatric disturbances (one depression and one anxiety). Data about previous-month sleep and mood, stress, anxiety trait, and resilience were obtained at baseline (**Table 3**). The mean score at STAI-Y2-*trait* was 34.5 ± 7.7, which is in the normal range, but eight participants (22.2%) showed increased values above threshold according to age and gender (according to the Italian normative data, Pedrabissi and Santinello, 1989). Mean PSQI score was 4.4 ± 2.6, nonetheless eight participants (22.2%) were poor sleepers (mostly related to the night shifts). Mean HADS-A (anxiety; 4.2 ± 2.9) and HADS-D (depression; 1.5 ± 2.0) scores were normal (<8), but five (13.9%) participants showed a value above threshold in HADS-A while no abnormal values were observed in the HADS-D. Moderate perception of stress was present in 12 participants (33.3%), and this was referred as related to the job workload. All the participants seemed to have a good resilience (score > 64; the ability to recover quickly from difficult and potentially harmful situations; Fletcher and Sarkar, 2013). None of the participants dropped out.

Physiological Parameters, Questionnaires, and LLS

Physiological values (SpO_2 and HR) along with the LLS, ISI, and STAI-Y1-*state* obtained across all four assessments are shown in **Figure 2**. SpO_2 decreased and HR increased with acute HH exposure. LLS increased at altitude arrival ($p = 0.015$) and four participants complained of AMS (LLS 5, 3, 3, and 3) after the first night at altitude. LLS decreased after the second night at altitude returning to the baseline level ($p < 0.001$). ISI was higher after the first night at altitude (3.9 ± 3.5 vs. 6.4 ± 4.1, $p = 0.001$) but returned to the baseline level after the second night (6.4 ± 4.1 vs. 3.6 ± 3.6, $p = 0.001$). Mean values for the anxiety state measured with STAI-Y1-*state* decreased at altitude; however, the reduction was significantly different from the baseline only at sessions 2 and 3 (29.3 ± 6.6 vs. 27.0 ± 5.4, $p = 0.033$ vs. 26.9 ± 4.8; $p = 0.032$).

Cognitive Tests (DSST, BART, and PVT)

The number of correct responses on the DSST decreased during the first 12 h at altitude (48.4 ± 6.2 vs. 44.8 ± 8.0, $p = 0.009$) and increased again after the second night at altitude (50.5 ± 6.7 in session 4, $p < 0.001$ for comparison with the session 2) (**Table 4**; **Figure 3**). GEE analysis showed no effect of altitude on the number of incorrect responses on DSST ($p = 0.253$).

TABLE 4 | *P*-values of effects estimated by generalized estimating equations (GEE).

Test	Parameter	Session	Gender	Age	Test sequence	LLS ≥ 3 at session 2 or session 3	ISI	SpO_2	Session * Gender
BART	Total time[#]	**<0.001**	1.000	1.000	1.000	1.000	1.000	0.083	1.000
	Mean earnings per balloon	**0.044**	1.000	1.000	1.000	1.000	0.668	1.000	1.000
	Mean pumps per balloon	0.055	1.000	1.000	1.000	1.000	0.790	1.000	1.000
DSST	Number of correct trials	**<0.001**	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	Number of incorrect trials	0.253	1.000	1.000	0.194	1.000	0.395	0.263	0.607
PVT	Number of trials with reaction time > 355 ms	1.000	1.000	1.000	1.000	1.000	1.000	0.843	1.000
	Number of false starts	0.103	1.000	1.000	1.000	0.205	0.045	0.420	1.000
	Mean reaction time of correct trials ≤ 355 ms	1.000	0.627	0.105	1.000	1.000	1.000	1.000	1.000

An asterisk between two factors indicates the effect of interaction of the two factors. Statistically significant values are reported in bold. BART, Balloon Analogue Risk Taking; DSST, Digit Symbol Substitution Test; ISI, Insomnia Severity Index; LLS, Lake Louise Score; PVT, Psychomotor Vigilance Test; SpO_2, peripheral oxygen saturation. [#]One case excluded from the analysis because outlier.

BART total time of test execution was faster during the last session (190.4 ± 39.0 ms) in comparison to the first three (218.4 ± 44.1, 212.1 ± 52.6, and 200.9 ± 34.3 ms; $p = 0.018$, $p = 0.001$, and $p = 0.035$, respectively). BART mean earnings per balloon were slightly higher after the second night at altitude in comparison to the first session at altitude (10.1 ± 0.9 vs. 9.8 ± 0.9, $p = 0.011$) and to the session after the first night at altitude (10.1 ± 0.9 vs. 9.9 ± 0.9, $p = 0.035$). BART mean pumps per balloon did not change during the four sessions.

There was no effect of altitude on the parameters of the PVT (mean reaction time, number of lapses and number of false starts) but GEE showed an effect of ISI on the number of false starts ($p = 0.045$) as individuals with ISI higher than 7 made more false starts [1.5 (95% CI 1.0–2.2) vs. 0.9 (95% CI 0.6–1.3)].

No effect of gender on the cognitive tests was detected.

DISCUSSION

The main finding of this study on lowlanders after ascent to 3,269 m is that the acute exposure to HH induced impairment in oxygen saturation and produced changes in speed of processing (DSST) at arrival at altitude. There was a fairly rapid recovery since there were no more detectable effects after 36 h of exposure to HH. Psychomotor vigilance was unaffected at altitude except for individuals with poor sleep, and the BART total time of execution was faster on the last session compared to the first three, but it was not associated with clinically relevant lower performance and therefore, likely due to a learning effect.

Exposure to HH reduced SpO_2 and increased HR due to the reduction in barometric pressure, which physiologically activates peripheral chemoreceptors and therefore sympathetic adrenergic response (Richalet, 2016). Simultaneously to physiological changes, our data provide evidence of minimal cognitive impairment after an acute exposure to altitude (3,269 m) up to 36 h in both men and women. This result is in line with other studies that showed an impaired performance on the DSST at higher altitudes and with different study designs (Evans and Witt, 1966; Berry et al., 1989; Wang et al., 2013; Hu et al., 2016). Hu et al. (2016) showed a reduced score on DSST compared to the baseline score in 100 male military participants after one night at 3650 m; after 7 days they climb to 4,400 m and a further decrease of DSST score was observed after staying for 72 h at the same altitude (4,400 m). DSST increased again after 1 and 3 months of staying at altitude (Hu et al., 2016). This finding is in agreement with our results showing a cognitive impairment already after acute HH exposure (at arrival and after around 12 h). Wang et al. (2013) evaluated the effect of acetazolamide, used to prevent AMS, on neurocognitive performance in 21 male participants flying from Xianyang (402 m) to Lhasa (3,561 m). In this randomized, double-blind, placebo-controlled study, they observed a significant decline in the acetazolamide group in the DSST performed 6 h after arrival at altitude (but not 24 or 48 h later). Similar results were obtained by Berry et al. (1989) in 20 male individuals and by Evans and Witt (1966) in 16 male individuals using a hypobaric chamber (4,500 m). Our data suggest that even at altitudes below 3,500 m, there could be an increased risk in performing demanding activities the day after arrival at altitude due to a decreased processing speed. Differently from the other studies, we enrolled both male and female, but we did not find any difference based on gender.

We also observed a quick recovery within 36 h of the initial impairment on DSST while staying at altitude, suggesting a positive effect of acclimatization. Previous studies showed an improvement of such task even with a progressive gradual ascent at altitude. Harris et al. (2009) observed a significant improvement in the DSST in 26 individuals (female and male) after 18 days of ascent to 5,100 m, or Walsh et al. (2020) in 15 individuals after 7 days of trekking to altitude (4,240 m), with impairment after exercise at higher compared to lower altitude (Walsh et al., 2020). These results may be related to the ascent profile in-agreement with the recommended guidelines to prevent altitude illnesses (Luks et al., 2019), which allows for acclimatization and prevents any neurological effects of altitude. We showed that such adaptation can occur within 2–3 days at an altitude below 3,500 m.

DSST is a fairly unspecific task that, in general, evaluates speed of processing. As with all tests, it is subject to a learning

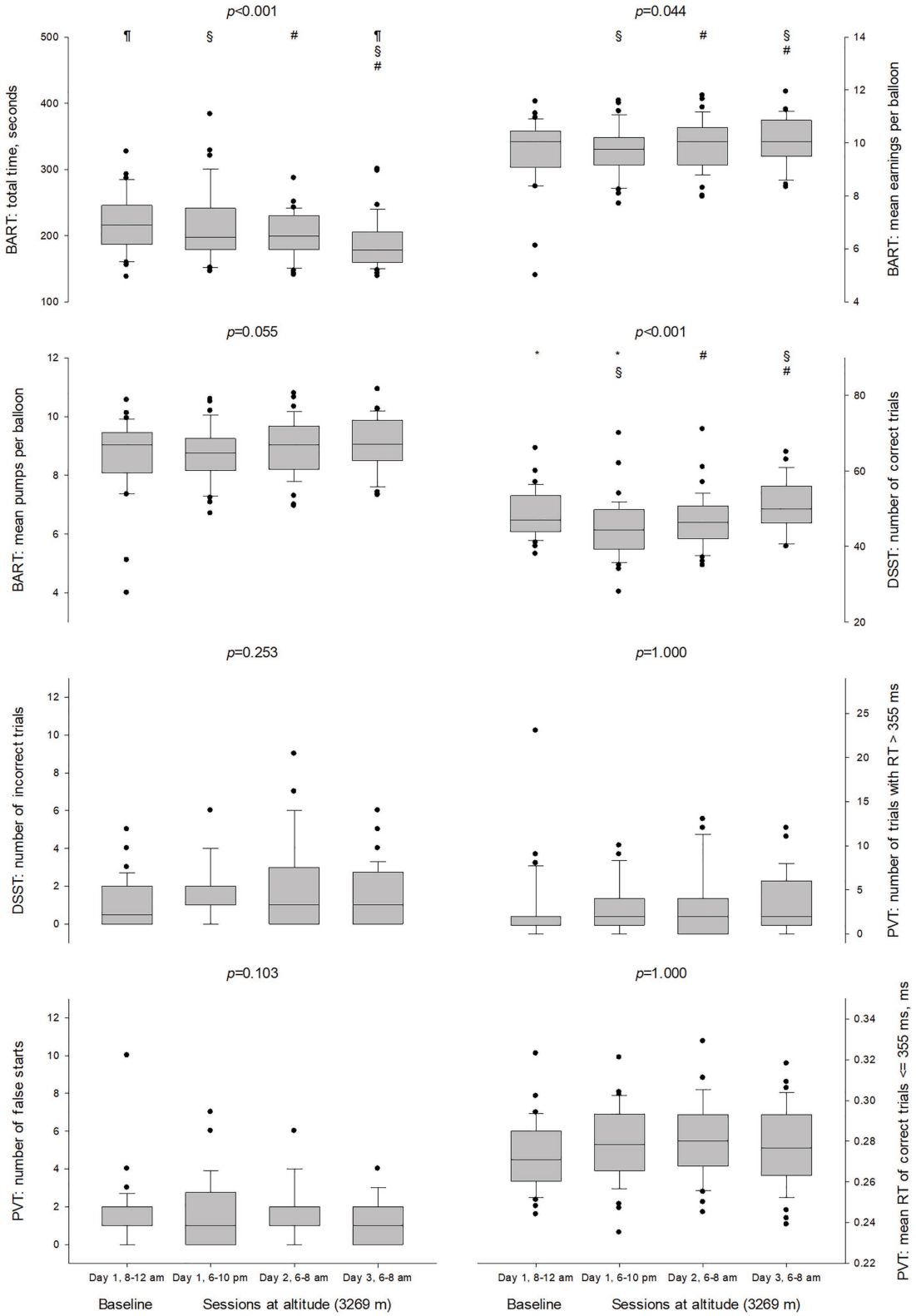

FIGURE 3 | Boxplots of cognitive test parameters at baseline and sessions at altitude (3,269 m). The *p*-values were calculated by means of generalized estimating equations (GEE) and adjusted by means of Holm-Bonferroni correction. Statistically significant ($p < 0.05$) pairwise comparisons were denoted by the following

(Continued)

FIGURE 3 | symbols: *for session 1 (day 1, 8:00–12:00 AM) vs. session 2 (day 1, 6:00–10:00 PM), ¶for session 1 vs. session 4 (day 3, 6:00–8:00 AM), §for session 2 vs. session 4, and #for session 3 (day 2, 6:00–8:00 AM) vs. session 4. BART, Balloon Analogue Risk Task; DSST, Digit Symbol Substitution Test; PVT, Psychomotor Vigilance Test; RT, reaction time; •, outlier.

effect (improvement over repeated administrations). We used parallel forms across the repeated administration to minimize this, but the effect is not explicitly discussed in most of the studies. DSST is also sensitive to the age effect (Hoyer et al., 2004), but our sample only included relatively young and well-educated individuals. DSST is highly sensitive to detect impairment but has low specificity in determining which cognitive domain is primarily involved. In our study, the psychomotor speed and the sustained attention were also measured with the PVT; our results showed no impairment on the PVT after HH exposure. Therefore, our results suggest that the main problem of the altitude reached in our study is a reduction in general ability, namely speed of processing, so that the same tasks can be equally performed but requires a longer time of execution.

Our results showed no effects on decision-making under ambiguity. Such results are in contrast to previous studies that investigate decision-making with the BART (Heinrich et al., 2019; Pighin et al., 2020). One possible explanation is that our study sample included only health care providers (medical doctors and nurses) who engage in decision-making activities, under stress, on a daily basis. Further research should consider populations with different characteristics. Moreover, while Heinrich et al. (2019) performed an in-field study similar to us with an exposure to HH (3,800 m), Pighin et al. (2020) performed the study in a normobaric hypoxia simulated environment (3,000 m).

Our study showed preserved psychomotor vigilance after HH exposure in line with the results of other studies performed below 4,000 m (Thomas et al., 2007; De Bels et al., 2019; Heinrich et al., 2019) but is in contrast with those performed above 4,000 m (Roach et al., 2014; Davranche et al., 2016; Pun et al., 2018). However, more false starts at the PVT were observed in individuals with a worse sleep quality measured with the ISI after the first night at altitude (ISI > 7).

Four individuals complained of AMS but there was no association with worse cognitive performance compared to other individuals.

Our findings are important because a large number of lowlanders often ascend rapidly to an altitude above 3,000 m for recreational and occupational purposes. It is known that altitude illnesses can occur during travel to elevations above 2,500 m (Paralikar and Paralikar, 2010). AMS and HACE usually present detectable signs and symptoms, whereas the reduction of cognitive performance is less perceived (Neuhaus and Hinkelbein, 2014). We confirm that an impairment of selective cognitive performance can appear even after an acute exposure to 3,269 m, while other cognitive aspects are preserved (i.e., decision-making and psychomotor vigilance). Furthermore, the speed of processing impairment that was observed during the first 24 h at HA was followed by an improvement 36 h after arrival. This is an important finding that may help to improve not only the safety of mountaineers, but also of altitude workers. We suggest a resting day before planning further ascent to higher altitudes or to perform risky activities for recreational or occupational purposes to prevent not only altitude illnesses, but also the risk of accidents.

Limitations

There are limitations worth noting. A limitation of this study was the absence of a time-matched low-altitude control group. Due to learning effects related to the repeated administration of cognitive tests, the inclusion of a control group would have been useful to isolate the altitude effect on cognitive function. Our sample was composed of relatively young individuals, and all were health-care providers, which may hamper the generalization of these findings to a broader population. However, we consider this group homogeneity selection as a strength of our study, which may broaden the application of these findings to health-care provider missions at this altitude (both rescue missions in wilderness environment reachable on foot and by helicopter). It is also uncertain whether the results would differ from those of other ethnic groups. Lastly, exhaustion was not evaluated, so we cannot say if the cognitive impairment after arrival at altitude was due solely to HH exposure or to a combination of physical effort and HH effect. Nevertheless, the persistence of the changes after a night of rest supports at least a partial effect of HH exposure per se.

CONCLUSION

Our study provides evidence of a reduced processing speed in lowlanders when exposed to altitude (3,269 m) in the first 24 h at altitude. There was a fairly quick recovery since it was no longer detectable after 36 h of exposure to HH. There were no clinically relevant effects on decision-making, while psychomotor vigilance was unaffected at altitude except for individuals with poor sleep. Further investigation in populations with different ethnical background and ages are warranted to confirm this observation and potentially guide the implementation of safety procedures at altitude.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Bolzano (Protocol Number 812020-BZ). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF, CP, AV, and GS contributed to the conception and design of the study. MF, AV, JK, SM-S, and GS performed the study. MF, JK, and TD organized the database. TD and MF performed the statistical analysis. MF, KH, EW, BW, MP, HB, and GS developed tools to perform the study. MF, CP, TD, JK, and GS drafted the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Basner, M., Mollicone, D., and Dinges, D. F. (2011). Validity and sensitivity of a brief psychomotor vigilance test (PVT-B) to total and partial sleep deprivation. *Acta Astronaut.* 69, 949–959. doi: 10.1016/j.actaastro.2011.07.015

Berry, D. T. R., McConnell, J. W., Phillips, B. A., Carswell, C. M., Lamb, D. G., and Prine, B. C. (1989). Isocapnic hypoxemia and neuropsychological functioning. *J. Clin. Exp. Neuropsychol.* 11, 241–251. doi: 10.1080/01688638908400886

Brodmann, M. M., Brugger, H., Pun, M., Strapazzon, G., Dal, C. T., Maggiorini, M., et al. (2018). The STAR data reporting guidelines for clinical high altitude research. *High Alt. Med. Biol.* 19, 7–14. doi: 10.1089/ham.2017.0160

Buysse, D. J., Reynolds, C. F. III., Monk, T. H., Berman, S. R., and Kupfer, D. J. (1989). The Pittsburgh sleep quality index: a new instrument for psychiatric practice and research. *Psychiatry Res.* 28, 193–213. doi: 10.1016/0165-1781(89)90047-4

Cohen, S., and Williamson, G. M. (1988). "Perceived Stress in a Probability Sample in the United States," in *The Social Psychology of Health*. eds. S. Spacapan and S. Oskamp (Newbury Park, CA: Sage), 31–67.

Davranche, K., Casini, L., Arnal, P. J., Rupp, T., Perrey, S., and Verges, S. (2016). Cognitive functions and cerebral oxygenation changes during acute and prolonged hypoxic exposure. *Physiol. Behav.* 164, 189–197. doi: 10.1016/j.physbeh.2016.06.001

De Bels, D., Pierrakos, C., Bruneteau, A., Reul, F., Crevecoeur, Q., Marrone, N., et al. (2019). Variation of cognitive function during a short stay at hypobaric hypoxia chamber (altitude: 3842 M). *Front. Physiol.* 10:806. doi: 10.3389/fphys.2019.00806

Evans, W. O., and Witt, N. F. (1966). The interaction of high altitude and psychotropic drug action. *Psychopharmacologia* 10, 184–188. doi: 10.1007/BF00455979

Fletcher, D., and Sarkar, M. (2013). Psychological resilience. *Eur. Psychol.* 18, 12–23. doi: 10.1027/1016-9040/a000124

Harris, G. A., Cleland, J., Collie, A., and McCrory, P. (2009). Cognitive assessment of a trekking expedition to 5100 m: a comparison of computerized and written testing methods. *Wilderness Environ. Med.* 20, 261–268. doi: 10.1580/08-WEME-OR-261R.1

Heinrich, E. C., Djokic, M. A., Gilbertson, D., DeYoung, P. N., Bosompra, N. O., Wu, L., et al. (2019). Cognitive function and mood at high altitude following acclimatization and use of supplemental oxygen and adaptive servoventilation sleep treatments. *PLoS One* 14:e0217089. doi: 10.1371/journal.pone.0217089

Hornbein, T. F., Townes, B. D., Schoene, R. B., Sutton, J. R., and Houston, C. S. (1989). The cost to the central nervous system of climbing to extremely high altitude. *N. Engl. J. Med.* 21, 1714–1719. doi: 10.1056/NEJM198912213212505

Hoyer, W. J., Stawski, R. S., Wasylyshyn, C., and Verhaeghen, P. (2004). Adult age and digit symbol substitution performance: a meta-analysis. *Psychol. Aging* 19, 211–214. doi: 10.1037/0882-7974.19.1.211

Hu, S. L., Xiong, W., Dai, Z. Q., Zhao, H. L., and Feng, H. (2016). Cognitive changes during prolonged stay at high altitude and its correlation with C-reactive protein. *PLoS One* 11:e0146290. doi: 10.1371/journal.pone.0146290

Jung, M., Zou, L., Yu, J. J., Ryu, S., Kong, Z., Yang, L., et al. (2020). Does exercise have a protective effect on cognitive function under hypoxia? A systematic review with meta-analysis. *J. Sport Health Sci.* 9, 562–577. doi: 10.1016/j.jshs.2020.04.004

Lejuez, C. W., Read, J. P., Kahler, C. W., Richards, J. B., Ramsey, S. E., Stuart, G. L., et al. (2002). Evaluation of a behavioral measure of risk taking: the balloon analogue risk task (BART). *J. Exp. Psychol. Appl.* 8, 75–84. doi: 10.1037/1076-898X.8.2.75

Li, X. Y., Wu, X. Y., Fu, C., Shen, X. F., Yang, C. B., and Wu, Y. H. (2000). Effects of acute exposure to mild or moderate hypoxia on human psychomotor performance and visual-reaction time. *Space Med. Med. Eng.* 13, 235–239.

Loprinzi, P. D., Matalgah, A., Crawford, L., Yu, J. J., Kong, Z., Wang, B., et al. (2019). Effects of acute normobaric hypoxia on memory interference. *Brain Sci.* 9:323. doi: 10.3390/brainsci9110323

Luks, A. M., Auerbach, P. S., Freer, L., Grissom, C. K., Keyes, L. E., McIntosh, S. E., et al. (2019). Wilderness medical society clinical practice guidelines for the prevention and treatment of acute altitude illness: 2019 update. *Wilderness Environ. Med.* 30, S3–S18. doi: 10.1016/j.wem.2019.04.006

McMorris, T., Hale, B. J., Barwood, M., Costello, J., and Corbett, J. (2017). Corrigendum to "Effect of acute hypoxia on cognition: a systematic review and meta-regression analysis." Neurosci. Biobehav. Rev. 74, 225-232. *Neurosci. Biobehav. Rev.* 98:333. doi: 10.1016/j.neubiorev.2019.01.017

Monasterio, M. E. (2005). Accident and fatality characteristics in a population of mountain climbers in New Zealand. *N. Z. Med. J.* 118:U1249.

Morin, C. M., Belleville, G., Bélanger, L., and Ivers, H. (2011). The insomnia severity index: psychometric indicators to detect insomnia cases and evaluate treatment response. *Sleep* 34, 601–608. doi: 10.1093/sleep/34.5.601

Neuhaus, C., and Hinkelbein, J. (2014). Cognitive responses to hypobaric hypoxia: implications for aviation training. *Psychol. Res. Behav. Manag.* 7, 297–302. doi: 10.2147/PRBM.S51844

Paralikar, S. J., and Paralikar, J. H. (2010). High-altitude medicine. *Indian J. Occup. Environ. Med.* 14, 6–12. doi: 10.4103/0019-5278.64608

Pavlicek, V., Schirlo, C., Nebel, A., Regard, M., Koller, E. A., and Brugger, P. (2005). Cognitive and emotional processing at high altitude. *Aviat. Space Environ. Med.* 76, 28–33.

Pedrabissi, L., and Santinello, M. (1989). STAI State-Trait Anxiety Inventory Forma Y: Manuale. Organizzazioni Speciali, Firenze, Edizione italiana: Luigi Pedrabissi e Massimo Santinello, Giunti OS.

Pighin, S., Bonini, N., Hadjichristidis, C., Schena, F., and Savadori, L. (2020). Decision making under stress: mild hypoxia leads to increased risk-taking. *Stress* 23, 290–297. doi: 10.1080/10253890.2019.1680634

Pun, M., Hartmann, S. E., Furian, M., Dyck, A. M., Muralt, L., Lichtblau, M., et al. (2018). Effect of acute, subacute, and repeated exposure to high altitude (5050 m) on psychomotor vigilance. *Front. Physiol.* 9:677. doi: 10.3389/fphys.2018.00677

Richalet, J. P. (2016). Physiological and clinical implications of adrenergic pathways at high altitude. *Adv. Exp. Med. Biol.* 903, 343–356. doi: 10.1007/978-1-4899-7678-9_23

Roach, E. B., Bleiberg, J., Lathan, C. E., Wolpert, L., Tsao, J. W., and Roach, R. C. (2014). AltitudeOmics: decreased reaction time after high altitude cognitive testing is a sensitive metric of hypoxic impairment. *Neuroreport* 25, 814–818. doi: 10.1097/WNR.0000000000000169

Roach, R. C., Hackett, P. H., Oelz, O., Bärtsch, P., Luks, A. M., MacInnis, M. J., et al. (2018). The 2018 Lake Louise Acute Mountain sickness score. *High Alt. Med. Biol.* 19, 4–6. doi: 10.1089/ham.2017.0164

Spielberger, C. D., Gorsuch, R. L., Lushene, R., Vagg, P. R., and Jacobs, G. A. (1983). *Manual for the State-Trait Anxiety Inventory*. Palo Alto, CA: Consulting Psychologists Press.

Taylor, A. (2011). High-altitude illnesses: physiology, risk factors, prevention, and treatment. *Rambam Maimonides Med. J.* 2:e0022. doi: 10.5041/RMMJ.10022

Thomas, R. J., Tamisier, R., Boucher, J., Kotlar, Y., Vigneault, K., Weiss, J. W., et al. (2007). Nocturnal hypoxia exposure with simulated altitude for 14 days

does not significantly alter working memory or vigilance in humans. *Sleep* 30, 1195–1203. doi: 10.1093/sleep/30.9.1195

van Alem, A. P., de Vos, R., Schmand, B., and Koster, R. W. (2004). Cognitive impairment in survivors of out-of-hospital cardiac arrest. *Am. Heart J.* 148, 416–421. doi: 10.1016/j.ahj.2004.01.031

Wagnild, G. (2009). *The Resilience Scale User's Guide for the US Englishversion of the Resilience Scale and the 14-Item Resilience Scale (RS-14)*. Worden, MT: Resilience Center.

Wagnild, G. M., and Young, H. M. (1993). Development and psychometric evaluation of the resilience scale. *J. Nurs. Meas.* 1, 165–178.

Walsh, J. J., Drouin, P. J., King, T. J., D'Urzo, K. A., Tschakovsky, M. E., Cheung, S. S., et al. (2020). Acute aerobic exercise impairs aspects of cognitive function at high altitude. *Physiol. Behav.* 223:112979. doi: 10.1016/j.physbeh.2020.112979

Wang, J., Ke, T., Zhang, X., Chen, Y., Liu, M., Chen, J., et al. (2013). Effects of acetazolamide on cognitive performance during high-altitude exposure. *Neurotoxicol. Teratol.* 35, 28–33. doi: 10.1016/j.ntt.2012.12.003

Wechsler, D. (2008). *Wechsler Adult Intelligence Scale: WAIS-IV; Technical and Interpretive Manual*. San Antonio, Tex: Pearson.

Zigmond, A. S., and Snaith, R. P. (1983). The hospital anxiety and depression scale. *Acta Psychiatr. Scand.* 67, 361–370. doi: 10.1111/j.1600-0447.1983.tb09716.x

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Physiological Responses to Hypoxia on Triglyceride Levels

*Renée Morin[1], Nicholas Goulet[1], Jean-François Mauger[1] and Pascal Imbeault[1,2]**

[1] School of Human Kinetics, Faculty of Health Sciences, University of Ottawa, Ottawa, ON, Canada, [2] Hôpital Montfort, Institut du Savoir Montfort, Ottawa, ON, Canada

Hypoxia is a condition during which the body or specific tissues are deprived of oxygen. This phenomenon can occur in response to exposure to hypoxic environmental conditions such as high-altitude, or because of pathophysiological conditions such as obstructive sleep apnea. Circumstances such as these can restrict supply or increase consumption of oxygen, leading to oxyhemoglobin desaturation and tissue hypoxia. In certain cases, hypoxia may lead to severe health consequences such as an increased risk of developing cardiovascular diseases and type 2 diabetes. A potential explanation for the link between hypoxia and an increased risk of developing cardiovascular diseases lies in the disturbing effect of hypoxia on circulating blood lipids, specifically its capacity to increase plasma triglyceride concentrations. Increased circulating triglyceride levels result from the production of triglyceride-rich lipoproteins, such as very-low-density lipoproteins and chylomicrons, exceeding their clearance rate. Considerable research in murine models reports that hypoxia may have detrimental effects on several aspects of triglyceride metabolism. However, in humans, the mechanisms underlying the disturbing effect of hypoxia on triglyceride levels remain unclear. In this mini-review, we outline the available evidence on the physiological responses to hypoxia and their impact on circulating triglyceride levels. We also discuss mechanisms by which hypoxia affects various organs involved in the metabolism of triglyceride-rich lipoproteins. This information will benefit scientists and clinicians interested in the mechanistic of the regulatory cascade responsible for the response to hypoxia and how this response could lead to a deteriorated lipid profile and an increased risk of developing hypoxia-related health consequences.

Keywords: intermittent hypoxia, continuous hypoxia, dyslipidemia, triglycerides, triglyceride-rich lipoproteins

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***Correspondence:**
Pascal Imbeault
imbeault@uottawa.ca

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INTRODUCTION

Through evolution, organisms have developed physiological systems to maintain and regulate oxygen (O_2) homeostasis (Semenza, 2000). O_2 plays the most important role in vegetal and animal respiration serving as the electron acceptor during oxidative phosphorylation (Brahimi-Horn and Pouysségur, 2007). Hypoxia is a condition during which the body is deprived of adequate O_2 at

Abbreviations: AT, Adipose tissue; ATGL, Adipose triglyceride lipase; CM, Chylomicron; CPAP, Continuous positive airway pressure; CVD, Cardiovascular disease; FiO_2, Fraction of inspired oxygen; HIF, Hypoxia-inducible factor; HSL, Hormone-sensitive lipase; IH, Intermittent hypoxia; LPL, Lipoprotein lipase; MUFA, Monounsaturated fatty acid; NEFA, Non-esterified fatty acid; O_2, Oxygen; OSA, Obstructive sleep apnea; SCD-1, Stearoyl–coenzyme A desaturase 1; SNS, Sympathetic nervous system; SREPB-1, Sterol regulatory element-binding protein 1; TG, Triglyceride; TRL, Triglyceride-rich lipoprotein; VLDL, Very-low-density lipoprotein.

the tissue level. Essentially, hypoxia occurs when O_2 demand is greater than O_2 delivery, initiating changes in gene expression mediated by a class of transcriptional factors called hypoxia-inducible factors (HIF). HIF-1, one of 3 major HIF isoforms, is known as the master regulator of cellular responses to hypoxia and activates the transcription of more than 1,000 genes encoding enzymes, as well as transport and mitochondrial proteins controlling the delivery and utilization of O_2 (Semenza, 2014). Environmental conditions such as high altitude, or pathological conditions such as obstructive sleep apnea (OSA) and chronic obstructive pulmonary disease can lead to tissue hypoxia (Raguso et al., 2004; Drager et al., 2010). In some conditions (e.g., OSA), individuals exposed to hypoxia are at an increased risk of developing cardiovascular diseases (CVD) and metabolic disorders (Drager et al., 2013). A potential explanation linking hypoxia to these health consequences lies in its disturbing effect on lipid metabolism. More precisely, hypoxia may disturb the balance between lipid storage and lipid mobilization in hepatic and adipose tissues (AT). These disturbances, characterized by an overproduction and/or an impaired clearance of triglyceride-rich lipoproteins (TRL), can lead to a deterioration of the blood lipid profile, specifically an increase in plasma triglyceride (TG) concentrations. Prolonged impairment of lipid storage and/or mobilization and overexposure of non-adipose tissue to high plasma lipid concentrations can in the long-term lead to lipotoxicity, which could promote the development of metabolic disorders (Lewis et al., 2002). Consequently, considerable research has investigated the effect of acute or chronic hypoxia on circulating TG levels. The following sections will provide key information on TG metabolism, experimental evidence linking hypoxia and circulating TG levels, and the main underlying mechanisms by which hypoxia modulates TG levels.

IMPORTANCE, PRODUCTION, AND EFFECTS OF TRIGLYCERIDES

TG are the most energy-dense substrate. They can be derived from food or produced through the conversion of excess carbohydrates and amino acids into fatty acids, through a process called *de novo* lipogenesis, that are then esterified to glycerol to produce TG. TG are composed of 3 fatty acids and 1 glycerol, and the long aliphatic chains provided by the fatty acids confers a highly hydrophobic character to the molecule. The transportation of TG in the aqueous environment typical of living organisms thus requires the help of specialized proteins called lipoproteins. Blood TG are secreted in the circulation in the form of TRL by the liver [as very-low-density lipoproteins (VLDL)] and the intestine [as chylomicrons (CM)] (Chapman et al., 2011; Packard et al., 2020). Humans ingest approximately 30–150 g of TG per day (Dubois et al., 1998). The small intestine's main roles include the digestion, absorption, and finally the secretion of dietary fats into the circulation in the form of CM (Xiao et al., 2011). In humans, a minimal consumption of 15 g of lipids is needed to induce a significant increase in postprandial CM levels (Dubois et al., 1998). Peak TG levels typically occur 2–4 h following meal ingestion. In the fasted state, normal TG levels should be lower than 1.7 mmol/L; higher values represent a CVD risk factor (Miller et al., 2011). It should also be noted that postprandial TG levels are superior to fasting TG as predictors of CVD risk (Kolovou and Ooi, 2013). Elevated triglyceridemia, both in fasting and postprandial states, is therefore increasingly considered a risk factor for CVD (Kolovou and Ooi, 2013).

TRIGLYCERIDE METABOLISM

Circulating TG levels reflect the balance between the production of TRL by the liver (as VLDL) and the intestine (as CM), and the disposal of TRL and their remnants by the periphery and the liver (Chapman et al., 2011; Packard et al., 2020; **Figure 1**). Following the ingestion of at least 15 g of lipids, a transient increase in TG levels and a change in plasma lipoprotein pattern occur (Lopez-Miranda et al., 2007). In brief, both the plasma concentrations of CM of intestinal origin and VLDL remnants increase (Nakajima et al., 2011). In contrast, in the postabsorptive state, TG are predominantly produced by the liver as VLDL. Both TRL are mainly hydrolyzed by the endothelium-bound enzyme lipoprotein lipase (LPL) which is secreted by AT and other tissues. Following the hydrolysis of VLDL and CM, low-density lipoprotein and CM remnants are metabolized by the liver to resynthesize VLDL (**Figure 1**). The secretion of VLDL by the liver is modulated according to the hormonal/nutritional state. During postabsorptive status or short-term fasting, the activation of the sympathetic nervous system (SNS) and the secretion of stress hormones facilitate the intracellular breakdown of TG within AT, which favor the efflux of non-esterified fatty acids (NEFA) into the circulation (Lafontan and Langin, 2009; Young and Zechner, 2013). NEFA are the main substrate for VLDL production (Xiao et al., 2011). Conversely, in postprandial or prandial states, insulin is secreted by the pancreas and strongly suppresses AT intracellular lipolysis, resulting in a reduced hepatic NEFA influx which represses hepatic VLDL production (Xiao et al., 2011; Nielsen and Karpe, 2012). Other non-negligible contributors to hepatic VLDL production are the *de novo* lipogenesis pathway and the uptake of TRL remnants. In summary, hepatic VLDL synthesis is mainly substrate-driven and primarily determined by hepatic lipid availability, to which NEFA delivery appears to be the main contributor (Nielsen and Karpe, 2012).

EXPERIMENTAL EVIDENCE LINKING HYPOXIA AND CIRCULATING TRIGLYCERIDE LEVELS

The following section discusses the results found in **Table 1**. For over half a century, hypoxia has been associated with lipemic disturbances (Louhija, 1969). **Table 1** summarizes experimental evidence from rodent and human studies reporting the effects of acute/chronic and intermittent/continuous hypoxia on AT-LPL activity and on circulating TG and NEFA concentrations. Studies were included if they simulated hypoxia in a controlled environment and/or had a weight-matched control group if the exposition time was longer than 1 day. Hypoxia is consistently

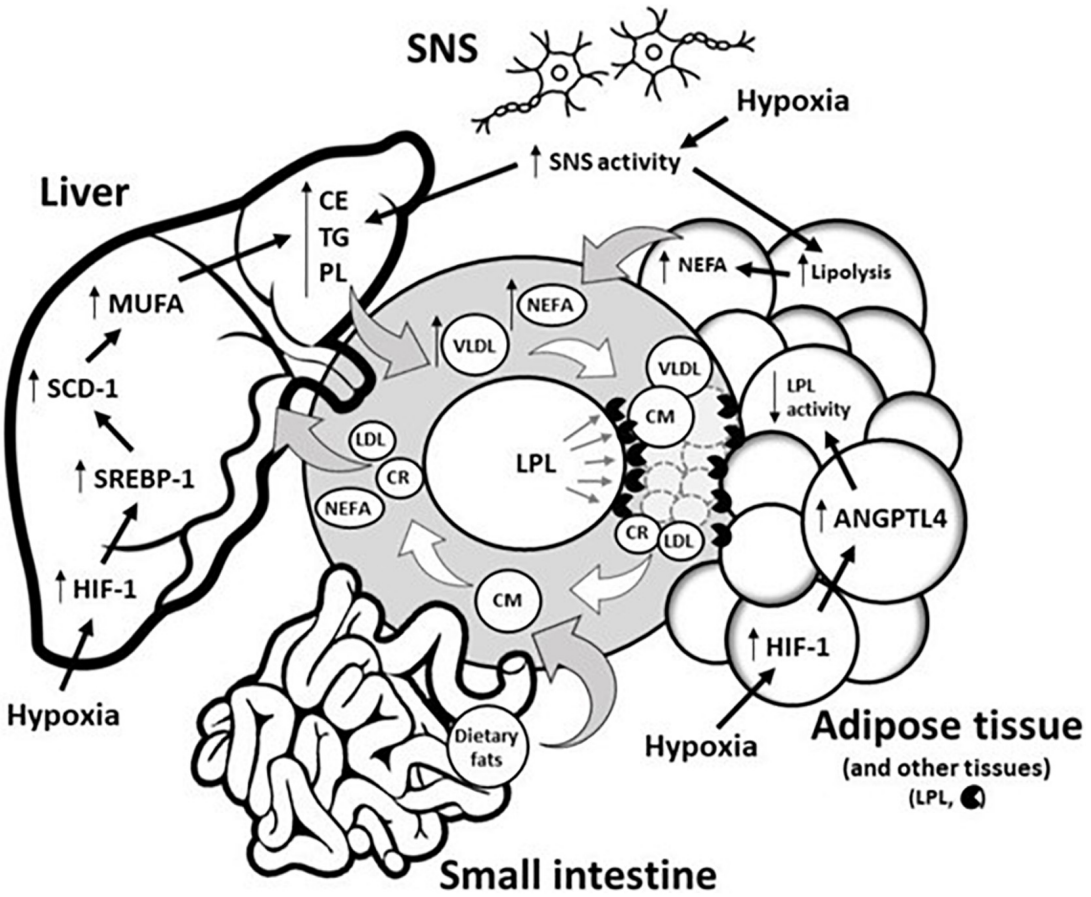

FIGURE 1 | Potential underlying physiological mechanisms by which hypoxia modulates blood triglyceride levels. Circulating TG levels reflect the production of TRL by both the liver (as VLDL) and the intestine (as CM) as well as the disposal of TRL and their remnants (LDL and CR) in the liver/periphery. Hypoxia does not disturb CM metabolism in the small intestine. Conversely, hypoxia disturbs hepatic and adipose tissue functions by (1) delaying hepatic removal of TRL remnants from circulation, and/or (2) delaying intravascular lipolysis of TRL via the HIF-1 upregulation of ANGTPL4, a potent inhibitor of adipose tissue LPL activity. In the liver, hypoxia also increases lipid biosynthesis via HIF-1-dependent upregulation of the transcriptional factor SREBP-1, which increases expression of SCD-1, the rate-limiting enzyme for the synthesis of MUFA, a major substrate for the synthesis of CE, TG, and PL. These latter substrates are key determinants involved in VLDL production. Through sympathetic activation, hypoxia can also increase VLDL production by (1) directly increasing VLDL assembly in the liver, and/or (2) by stimulating intracellular lipolysis of adipose tissue, which results in the increased release of NEFA, the main precursor to VLDL production. ANGPTL4, Angiopoietin-like 4; CM, Chylomicron; CR, Chylomicron remnant; CE, Cholesterol ester; HIF-1, Hypoxia-inducible factor 1; LDL, Low-density lipoprotein; LPL, Lipoprotein lipase; MUFA, Monounsaturated fatty acid; NEFA, Non-esterified fatty acid; PL, Phospholipid; SCD-1, Stearoyl-CoA desaturase-1; SREBP-1, Sterol regulatory element-binding protein 1; SNS, Sympathetic nervous system; TG, Triglyceride; VLDL, Very-low-density lipoprotein.

associated with elevated TG levels in murine models, with 12 out of 14 studies reporting an increase ranging from 20 to 570%, and no studies reporting a decrease. This wide range of increased TG levels could stem from several factors, including the type and severity of hypoxic exposure, diet, exposition time, nutritional status, temperature, and inter-species differences. Most notably, a negative relationship between the fraction of inspired oxygen (FiO_2) and TG levels has been observed (Jun et al., 2012).

A major concern regarding the physiological relevance of studies conducted with rodents is the effect of thermoneutrality. Jun et al. (2013) compared the effect of acute continuous hypoxia in mice exposed to either a thermoneutral condition (30°C) or a non-thermoneutral condition (22°C). They showed that cooler ambient temperatures increased lipid uptake in several tissues (e.g., liver, lungs, heart, brown, and white adipose tissues) and that hypoxia attenuated this stimulation, resulting in increased TG levels. Under the thermoneutral condition, no difference was observed in the lipid uptake of these tissues, and TG levels were not altered following 6 h of normoxia vs. hypoxia (FiO_2 = 0.10) exposure. These findings led the authors to conclude that ambient temperatures must be carefully considered in the design and interpretation of experiments that involve hypoxia in mice. This statement likely applies to experiments with humans given the well-recognized effects of cooler temperature on energy metabolism (van Marken Lichtenbelt et al., 2002; Blondin et al., 2017). Throughout the last couple of decades, rodent studies have also identified other factors that can weaken the disturbing effect of hypoxia on lipid metabolism. Those factors include, but are not limited to, pre-existing obesity (Li et al., 2005) and deficiencies in certain nuclear factors/proteins

TABLE 1 | Effects of acute/chronic and intermittent/continuous hypoxia on adipose tissue LPL activity, and on circulating levels of triglycerides and non-esterified fatty acids in rodent and human studies.

References	Year	IH/CH	Exposition time	Hypoxic severity	Adipose tissue LPL activity	Blood triglycerides	Blood non-esterified fatty acids	Nutritional status	Rodent/Human
Louhija (1969)	1969	CH	12 days	FiO_2 = 0.10	N/A	↑ 570%	No differences	Fasting	Sprague-Dawley rats
Férézou et al. (1993)	1993	CH	7 days	FiO_2 = 0.12	N/A	No differences	N/A	Fasting	Healthy young participants
					N/A	↓ 40%	N/A	Postprandial	Healthy young participants
Leaf and Kleinman (1996)	1996	CH	2 h	FiO_2 = 0.16	N/A	No differences	N/A	Fasting	Older men
Muratsubaki et al. (2003)	2003	CH	5 h	FiO_2 = 0.10	N/A	↑ 210%	N/A	Sated	Sprague-Dawley rats
					N/A	No differences	N/A	Fasting	Sprague-Dawley rats
Li et al. (2005)	2005	IH	5 days	60 cycles/h FiO_2 = 0.05	N/A	↑ 40%	No differences	Fasting	C57bl/6j mice, not thermoneutral, lean
					N/A	No differences	No differences	Fasting	C57bl/6j mice, not thermoneutral, obese
Li et al. (2007a)	2007	IH	4 weeks	FiO_2 = 0.05	N/A	↑ 25%	N/A	Fasting	C57bl/6j mice, not thermoneutral,
				FiO_2 = 0.10	N/A	No differences	N/A	Fasting	C57bl/6j mice, not thermoneutral,
Li et al. (2007b)	2007	IH	4 weeks	60 cycles/h FiO_2 = 0.05	N/A	↑ 100%	No differences	Fasting	Scap*fl/fl* mice, not thermoneutral
					N/A	No differences	No differences	Fasting	L-Scap- mice, not thermoneutral, deficiency in SREBP
Savransky et al. (2007)	2007	IH	12 weeks	60 cycles/h FiO_2 = 0.05	N/A	No differences	N/A	Fasting	C57bl/6j mice, not thermoneutral, regular diet
					N/A	No differences	N/A	Fasting	C57bl/6j mice, not thermoneutral, high cholesterol diet
Savransky et al. (2008)	2008	IH	10 weeks	60 cycles/h FiO_2 = 0.05	N/A	No differences	No differences	Fasting	C57bl/6j mice, not thermoneutral
					N/A	No differences	No differences	Fasting	C57bl/6j mice, not thermoneutral, treated with antisense SCD-1 oligonucleotides
Jun et al. (2010)	2010	IH	4 weeks	60 cycles/h FiO_2 = 0.07	N/A	↑ 60%	↑ 45%	Fasting	ApoE-/- mice, not thermoneutral
Drager et al. (2012)	2012	IH	4 weeks	60 cycles/h FiO_2 = 0.07	↓ 80%	↑ 100%	↓ 15% *	Fasting	C57bl/6j mice, not thermoneutral
Jun et al. (2012)	2012	CH	6 h	FiO_2 = 0.07	↓ 60%	↑ 150%	↑ 60%	Fasting	C57bl/6j mice, not thermoneutral
Jun et al. (2013)	2013	CH	6 h	FiO_2 = 0.10	↓ 60%	↑ 50%	↓ 40%	Fasting	C57bl/6j mice, not thermoneutral
					↓ 80%	No differences	No differences	Fasting	C57bl/6j mice, thermoneutral
Drager et al. (2013)	2013	IH	4 weeks	60 cycles/h FiO_2 = 0.07	N/A	↑ 55%	↑ 30%	Fasting	ApoE-/- mice, not thermoneutral
					N/A	No differences	↑ 35%	Fasting	ApoE-/- mice, not thermoneutral, treated with ANGPTL4-neutralizing antibody
Yao et al. (2013)	2013	IH	4 weeks	60 cycles/h FiO_2 = 0.07	↓ 50%	↑ 20%	N/A	Fasting	C57bl/6j mice, not thermoneutral
					No differences	↑ 75%	N/A	Fasting	C57bl/6j mice, not thermoneutral, treated with ANGTPL4-neutralizing antibody
Siques et al. (2014)	2014	CH	30 days	FiO_2 = 0.12	N/A	↑ 35%	N/A	Fasting	Winstar rats, not thermoneutral
Mahat et al. (2016)	2016	IH	6 h	17 cycles/h N_2 = 100%	No differences	No differences	↑ 25%	Postprandial	Healthy young men
Chopra et al. (2017)	2017	IH	3 nights	CPAP withdrawal	N/A	No differences	↑	Postprandial	OSA patients
Mahat et al. (2018)	2018	CH	6 h	FiO_2 = 0.12	N/A	No differences	↑ 95%	Fasting	Healthy young men
Mauger et al. (2019)	2019	CH	6 h	FiO_2 = 0.12	N/A	↑ 15% *	↑ 30%	Prandial	Healthy young men
Morin et al. (2021)	2021	IH	6 h	15 cycles/h N_2 = 100%	N/A	↑ 45%	↑ 25% *	Postprandial	Healthy young men
					N/A	No differences	No difference	Postprandial	OSA patients

*ANGPTL4, Angiopoietin-like 4; CH, continuous hypoxia; FiO_2, fraction of inspired oxygen; IH, intermittent hypoxia; LPL, lipoprotein lipase; OSA, obstructive sleep apnea; SCD-1, Stearoyl-CoA desaturase-1; SREBP-1, Sterol regulatory element-binding protein 1. *Trend (p-value between 0.05 and 0.1).*

(Li et al., 2007a; Savransky et al., 2008; Drager et al., 2013; Yao et al., 2013).

Human studies do not report elevated TG levels in response to hypoxia as consistently as in murine studies. Leaf and Kleinman (1996) observed no changes in fasting TG levels in individuals (mean age = 70 years) who underwent 2 h of continuous hypoxia exposure. More recently, Mahat et al. (2018) investigated the effect of a 6-h exposure to normobaric continuous hypoxia (FiO_2 = 0.12) in fasted healthy young men and reported no difference in TG levels. The same group also investigated the effect of acute intermittent hypoxia (IH) on postprandial TG levels in healthy young men in 2 independent studies with conflicting results despite using similar IH protocols. In one study, no difference in postprandial TG levels between IH and normoxia exposure was observed (Mahat et al., 2016), while in the other, a marginal transient increase in postprandial TG levels was observed after 3 h of IH exposure (Morin et al., 2021). This discrepancy is not readily explainable, but we speculate that it could be the result of higher postprandial peaks in TG levels achieved in the latter study. A study conducted in a steady prandial state provided further insight into the effect of hypoxia on lipid metabolism by showing a trend toward 15% higher prandial TG levels after 4 h of normobaric continuous hypoxia (Mauger et al., 2019).

The effect of hypoxia on circulating lipids has also been examined in pathophysiological conditions such as OSA, a sleep disorder characterized by episodes of partial or complete obstruction of the upper respiratory airways that leads to IH during sleep (Dempsey et al., 2010). Chopra et al. (2017) compared individuals with OSA, untreated or treated using continuous positive airway pressure (CPAP) withdrawal. CPAP withdrawal increased nocturnal NEFA but not TG levels. In line with this observation, Morin et al. (2021) recently showed no difference in postprandial TG in individuals with OSA exposed to 6 h of IH or ambient air, in a crossover design.

In summary, there is a consistent increase in TG levels in response to hypoxia in rodents while such a response is less obvious in human studies (**Table 1**). It is critical to keep in mind that the effect of thermoneutrality on circulating TG levels may be an important concern regarding the physiological relevance of studies conducted with rodents. In humans, studies have always been conducted in thermoneutrality. Would conducting human trials in non-thermoneutral conditions lead to similar inter-species results? This warrants further exploring. In rodents, it has been shown that hypoxia increases TG levels through two major mechanisms: increased hepatic secretion of VLDL (Li et al., 2007a) and decreased TRL clearance (Jun et al., 2012; Drager et al., 2013). In human studies, cellular and molecular mechanisms are still poorly understood and require further attention. Despite progress and recent developments, several questions remain, including the effects of age, sex, as well as the exact hypoxic threshold (severity × time) needed to trigger modulations of TG levels. The sections below elaborate on the knowledge to date pertaining to the underlying mechanisms by which hypoxia modulates circulating TG levels.

UNDERLYING MECHANISMS BY WHICH HYPOXIA MODULATES TRIGLYCERIDES LEVELS

White Adipose Tissue

White AT has an important buffering action on circulating lipids concentrations (e.g., NEFA and TG contained in lipoproteins) by modulating (1) the release of NEFA into circulation, which occurs through intracellular lipolysis, and (2) the clearance of TRL-TG, which occurs through intravascular lipolysis of TRL's content. The effects of hypoxia on these crucial pathways are discussed in this section.

Intracellular Lipolysis

In periods of food deprivation and/or excess metabolic need, TG stored in AT can be rapidly mobilized into the circulation as NEFA and glycerol. The catabolic process of intracellular lipolysis relies on 3 hydrolases, of which 2, namely the hormone-sensitive lipase (HSL) and the adipose TG lipase (ATGL), are considered regulated and rate-limiting (Lafontan and Langin, 2009; Young and Zechner, 2013). The HSL and ATGL are mainly under hormonal control with catecholamines and natriuretic peptides stimulating, and insulin inhibiting ATGL, HSL, and AT lipolysis. Hypoxia has been shown to increase SNS tone (Somers et al., 1989, 1995) and catecholamines secretion (Mesarwi et al., 2019). The increase in sympathetic activation in response to hypoxia exposure and its upregulation of intracellular lipolysis has elegantly been demonstrated by Weiszenstein et al. (2016). Specifically, they showed that IH exposure for 14 days in mice significantly increased adipocyte lipolysis and elevated NEFA levels, effects that were attenuated when mice were treated with a pharmacological lipolysis inhibitor, acipimox. In addition to its impact on AT intracellular lipolysis at the whole-body level via the activation of the SNS, hypoxia has also been shown to stimulate basal lipolysis of human subcutaneous (O'Rourke et al., 2013; Mahat et al., 2016, 2018) and visceral (O'Rourke et al., 2013) adipocytes. Cultured adipocytes in hypoxic conditions also showed an activation of the glucose transporter 1, the facilitative glucose transporter independent of insulin, and a reduction in insulin-dependent glucose transporter-4 (Regazzetti et al., 2009; Wood et al., 2011; Varela-Guruceaga et al., 2018). This state of insulin resistance in fat cells is dependent on HIF-1 transcription (Regazzetti et al., 2009; Varela-Guruceaga et al., 2018). Together, these findings provide evidence that hypoxia stimulates intracellular lipolysis and increases the release of NEFA into circulation, which likely explains the marked elevation in circulating NEFA levels commonly observed upon hypoxia exposure (**Table 1**).

Intravascular Lipolysis

Several tissues synthesize the enzyme LPL, the key intravascular lipolytic enzyme. Intracellularly produced LPL is secreted and transported to the luminal side of the capillary endothelium where it hydrolyzes TG contained in circulating TRL (Eckel, 1989; Kersten, 2014). The TG hydrolysis process releases NEFA, which can be taken up by nearby cells. In AT, this

cascade results in the intracellular re-esterification of NEFA into storage TG droplets. Therefore, AT-LPL plays a crucial role in the regulation of circulating TG as well as in the AT lipid storage capacity. As summarized in **Table 1**, rodent studies consistently reported attenuated AT-LPL activity in acute and chronic exposures to hypoxia, where the reduction ranges from 50 to 80% (Drager et al., 2012; Jun et al., 2012, 2013; Yao et al., 2013). In differentiated human preadipocytes, acute hypoxia has been shown to reduce LPL activity dose-dependently (Mahat et al., 2018) with a reduction up to sixfold after 24 h at a FiO_2 of 0.03 (Mahat et al., 2016). A well-recognized mechanism underlying the reduction of AT-LPL activity upon hypoxia exposure is the upregulation of angiopoietin-like 4 (Drager et al., 2012; Yao et al., 2013), a strong post-translational inhibitor of LPL activity (Wu et al., 2021) induced by HIF-1 (Drager et al., 2013). Together, these findings indicate that hypoxia alters the TG clearing capacity of AT.

Small Intestine

Ingested fat is the major regulator of CM production in the small intestine (Dash et al., 2015). As previously reviewed (Westerterp and Kayser, 2006; Kayser and Verges, 2013), energy digestibility measured by bomb calorimetry of feces is not affected by altitudes up to 6,500 m, an altitude that refers to a FiO_2 of $\sim$ 0.09. Using retinyl palmitate to study the dynamics of buoyant intestinal CM, it has previously been reported that mice exposed to chronic IH show no impairment of intestinal absorption as compared to mice exposed to normoxia (Drager et al., 2012). Recent evidence also suggests that the gastrointestinal tract seems to cope well with hypoxia exposure. More precisely, no difference was observed in the circulating CM-TG of healthy individuals after 6 h of normobaric hypoxia (FiO_2= 100%) after a meal (Morin et al., 2021).

Liver

The liver plays a major role in the regulation of TRL, primarily in VLDL production and TRL remnants disposal. VLDL production is modulated by (1) hepatic NEFA delivery, and (2) *de novo* lipogenesis (Nielsen and Karpe, 2012). Also, there is clear evidence that an increase in sympathetic tone to the liver directly leads to an increase in VLDL production (Geerling et al., 2014). As previously mentioned, the increase in sympathetic tone observed upon hypoxia favors both a release of NEFA into circulation, which serve as substrates for VLDL production, and a reduction in TG clearance. It has also been shown that IH, through activation of HIF-1, upregulates a key transcriptional factor involved in lipid biosynthesis, the sterol regulatory element-binding protein-1 (SREPB-1) (Li et al., 2005, 2006). Upregulation of SREPB-1 leads to an increase in the gene expression of several *de novo* lipogenic enzymes, including stearoyl–coenzyme A desaturase 1 (SCD-1) (Shimano, 2001), which facilitates the endogenous synthesis of monounsaturated fatty acids (MUFA). MUFA synthesized through this pathway serve as major substrates for the synthesis of cholesterol esters, TG, and phospholipids (Ntambi and Miyazaki, 2004) that are secreted in the circulation as lipoproteins if said production exceeds the liver's needs. Overall, hypoxia increases the key substrates involved in VLDL production.

A limited number of studies highlight the impact of hypoxia on the metabolism of TRL remnants. Drager et al. (2012) demonstrated that mice chronically exposed to IH have a decreased clearance of TRL. Interestingly, this effect was also shown in individuals living with severe OSA. Indeed, using radiolabeled lipids, Drager et al. (2018) observed that severe OSA delays both the lipolysis of TRL and the removal of TRL remnants from the circulation. They also demonstrated that the impairments in remnants removal and intravascular lipolysis were strongly correlated with the severity of nocturnal hypoxemia. More recently, a study reported that prandial TG concentrations associated with denser lipoproteins (CM remnants and VLDL) were increased by 6 h of normobaric hypoxia (Mauger et al., 2019). Acute IH was also shown to negatively affect postprandial TG levels in healthy individuals, due to an increase in denser TRL levels such as VLDL and CM remnants (Morin et al., 2021). These results support the concept that hypoxia disturbs the prandial/postprandial metabolism of denser TRL.

CONCLUSION

There is ample evidence from murine and human studies linking hypoxia with considerable adverse effects on TG metabolism and triglyceridemia through alterations in AT and liver functions. The present work highlights that hypoxia tends to negatively affect TG levels by increasing the concentration of denser TRL such as VLDL and CM remnants, an observation that mainly occurs in prandial and postprandial states. Taken together, these findings can help to orient future studies or develop strategies to alleviate the effect of hypoxia on TG levels, and therefore reduce the cardiovascular risk associated with hypoxia-inducing health conditions.

AUTHOR CONTRIBUTIONS

RM and NG drafted versions of the manuscript with input and revisions from PI. All authors edited, revised, and approved the final version of the manuscript.

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REFERENCES

Blondin, D. P., Tingelstad, H. C., Noll, C., Frisch, F., Phoenix, S., Guérin, B., et al. (2017). Dietary fatty acid metabolism of brown adipose tissue in cold-acclimated men. *Nat. Commun.* 8:14146. doi: 10.1038/ncomms14146

Brahimi-Horn, M. C., and Pouysségur, J. (2007). Oxygen, a source of life and stress. *FEBS Lett.* 581, 3582–3591. doi: 10.1016/j.febslet.2007.06.018

Chapman, M. J., Ginsberg, H. N., Amarenco, P., Andreotti, F., Borén, J., Catapano, A. L., et al. (2011). Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. *Eur. Heart J.* 32, 1345–1361. doi: 10.1093/eurheartj/ehr112

Chopra, S., Rathore, A., Younas, H., Pham, L. V., Gu, C., Beselman, A., et al. (2017). Obstructive sleep apnea dynamically increases nocturnal plasma free fatty acids, glucose, and cortisol during sleep. *J. Clin. Endocrinol. Metab.* 102, 3172–3181. doi: 10.1210/jc.2017-00619

Dash, S., Xiao, C., Morgantini, C., and Lewis, G. F. (2015). New insights into the regulation of chylomicron production. *Annu. Rev. Nutr.* 35, 265–294. doi: 10.1146/annurev-nutr-071714-034338

Dempsey, J. A., Veasey, S. C., Morgan, B. J., and O'Donnell, C. P. (2010). Pathophysiology of sleep Apnea. *Physiol. Rev.* 90, 47–112. doi: 10.1152/physrev.00043.2008

Drager, L. F., Jun, J., and Polotsky, V. Y. (2010). Obstructive Sleep Apnea and Dyslipidemia: implications for atherosclerosis. *Curr. Opin. Endocrinol. Diabetes Obes.* 17, 161–165. doi: 10.1097/MED.0b013e3283373624

Drager, L. F., Li, J., Shin, M.-K., Reinke, C., Aggarwal, N. R., Jun, J. C., et al. (2012). Intermittent hypoxia inhibits clearance of triglyceride-rich lipoproteins and inactivates adipose lipoprotein lipase in a mouse model of sleep apnoea. *Eur. Heart J.* 33, 783–790. doi: 10.1093/eurheartj/ehr097

Drager, L. F., Tavoni, T. M., Silva, V. M., Santos, R. D., Pedrosa, R. P., Bortolotto, L. A., et al. (2018). Obstructive sleep apnea and effects of continuous positive airway pressure on triglyceride-rich lipoprotein metabolism[S]. *J. Lipid Res.* 59, 1027–1033. doi: 10.1194/jlr.M083436

Drager, L. F., Yao, Q., Hernandez, K. L., Shin, M.-K., Bevans-Fonti, S., Gay, J., et al. (2013). Chronic intermittent hypoxia induces atherosclerosis via activation of adipose Angiopoietin-like 4. *Am. J. Respir. Crit. Care Med.* 188, 240–248. doi: 10.1164/rccm.201209-1688OC

Dubois, C., Beaumier, G., Juhel, C., Armand, M., Portugal, H., Pauli, A. M., et al. (1998). Effects of graded amounts (0–50 g) of dietary fat on postprandial lipemia and lipoproteins in normolipidemic adults. *Am. J. Clin. Nutr.* 67, 31–38. doi: 10.1093/ajcn/67.1.31

Eckel, R. H. (1989). Lipoprotein lipase. *N. Engl. J. Med.* 320, 1060–1068. doi: 10.1056/NEJM198904203201607

Férézou, J., Richalet, J. P., Sérougne, C., Coste, T., Wirquin, E., and Mathé, D. (1993). Reduction of postprandial Lipemia after acute exposure to high altitude hypoxia. *Int. J. Sports Med.* 14, 78–85. doi: 10.1055/s-2007-1021150

Geerling, J. J., Boon, M. R., Kooijman, S., Parlevliet, E. T., Havekes, L. M., Romijn, J. A., et al. (2014). Sympathetic nervous system control of triglyceride metabolism: novel concepts derived from recent studies. *J. Lipid Res.* 55, 180–189. doi: 10.1194/jlr.R045013

Jun, J., Reinke, C., Bedja, D., Berkowitz, D., Bevans-Fonti, S., Li, J., et al. (2010). Effect of intermittent hypoxia on atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* 209, 381–386. doi: 10.1016/j.atherosclerosis.2009.10.017

Jun, J. C., Shin, M.-K., Yao, Q., Bevans-Fonti, S., Poole, J., Drager, L. F., et al. (2012). Acute hypoxia induces hypertriglyceridemia by decreasing plasma triglyceride clearance in mice. *Am. J. Physiol. Endocrinol. Metab.* 303, E377–E388. doi: 10.1152/ajpendo.00641.2011

Jun, J. C., Shin, M.-K., Yao, Q., Devera, R., Fonti-Bevans, S., and Polotsky, V. Y. (2013). Thermoneutrality modifies the impact of hypoxia on lipid metabolism. *Am. J. Physiol. Endocrinol. Metab.* 304, E424–E435. doi: 10.1152/ajpendo.00515

Kayser, B., and Verges, S. (2013). Hypoxia, energy balance and obesity: from pathophysiological mechanisms to new treatment strategies. *Obes. Rev.* 14, 579–592. doi: 10.1111/obr.12034

Kersten, S. (2014). Physiological regulation of lipoprotein lipase. *Biochim. Biophys. Acta* 1841, 919–933. doi: 10.1016/j.bbalip.2014.03.013

Kolovou, G., and Ooi, T. C. (2013). Postprandial lipaemia and vascular disease. *Curr. Opin. Cardiol.* 28, 446–451. doi: 10.1097/HCO.0b013e3283606971

Lafontan, M., and Langin, D. (2009). Lipolysis and lipid mobilization in human adipose tissue. *Prog. Lipid Res.* 48, 275–297. doi: 10.1016/j.plipres.2009.05.001

Leaf, D. A., and Kleinman, M. T. (1996). Acute exposure to carbon monoxide does not affect plasma lipids, lipoproteins, and Apolipoproteins. *Angiology* 47, 337–341. doi: 10.1177/000331979604700403

Lewis, G. F., Carpentier, A., Adeli, K., and Giacca, A. (2002). Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr. Rev.* 23, 201–229. doi: 10.1210/edrv.23.2.0461

Li, J., Bosch-Marce, M., Nanayakkara, A., Savransky, V., Fried, S. K., Semenza, G. L., et al. (2006). Altered metabolic responses to intermittent hypoxia in mice with partial deficiency of hypoxia-inducible factor-1alpha. *Physiol. Genomics* 25, 450–457. doi: 10.1152/physiolgenomics.00293.2005

Li, J., Nanayakkara, A., Jun, J., Savransky, V., and Polotsky, V. Y. (2007a). Effect of deficiency in SREBP cleavage-activating protein on lipid metabolism during intermittent hypoxia. *Physiol. Genomics* 31, 273–280. doi: 10.1152/physiolgenomics.00082.2007

Li, J., Savransky, V., Nanayakkara, A., Smith, P. L., O'Donnell, C. P., and Polotsky, V. Y. (2007b). Hyperlipidemia and lipid peroxidation are dependent on the severity of chronic intermittent hypoxia. *J. Appl. Physiol.* 102, 557–563. doi: 10.1152/japplphysiol.01081.2006

Li, J., Thorne, L. N., Punjabi, N. M., Sun, C. K., Schwartz, A. R., Smith, P. L., et al. (2005). Intermittent hypoxia induces hyperlipidemia in lean mice. *Circ. Res.* 97, 698–706. doi: 10.1161/01.RES.0000183879.60089.a9

Lopez-Miranda, J., Williams, C., and Lairon, D. (2007). Dietary, physiological, genetic and pathological influences on postprandial lipid metabolism. *Br. J. Nutr.* 98, 458–473. doi: 10.1017/S000711450774268X

Louhija, A. (1969). Hypertriglyceridemia in rats at simulated high altitudes. *Experientia* 25, 248–249. doi: 10.1007/BF02034371

Mahat, B., Chassé, É., Lindon, C., Mauger, J.-F., and Imbeault, P. (2018). No effect of acute normobaric hypoxia on plasma triglyceride levels in fasting healthy men. *Appl. Physiol. Nutr. Metab.* 43, 727–732. doi: 10.1139/apnm-2017-0505

Mahat, B., Chassé, É., Mauger, J.-F., and Imbeault, P. (2016). Effects of acute hypoxia on human adipose tissue lipoprotein lipase activity and lipolysis. *J. Transl. Med.* 14:212. doi: 10.1186/s12967-016-0965-y

Mauger, J.-F., Chassé, É., Mahat, B., Lindon, C., Bordenave, N., and Imbeault, P. (2019). The effect of acute continuous hypoxia on triglyceride levels in constantly fed healthy men. *Front. Physiol.* 10:752. doi: 10.3389/fphys.2019.00752

Mesarwi, O. A., Loomba, R., and Malhotra, A. (2019). Obstructive sleep Apnea, hypoxia, and nonalcoholic fatty liver disease. *Am. J. Respir. Crit. Care Med.* 199, 830–841. doi: 10.1164/rccm.201806-1109TR

Miller, M., Stone, N. J., Ballantyne, C., Bittner, V., Criqui, M. H., Ginsberg, H. N., et al. (2011). Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. *Circulation* 123, 2292–2333. doi: 10.1161/CIR.0b013e3182160726

Morin, R., Mauger, J.-F., Amaratunga, R., and Imbeault, P. (2021). The effect of acute intermittent hypoxia on postprandial triglyceride levels in humans: a randomized crossover trial. *J. Transl. Med.* 19:268. doi: 10.1186/s12967-021-02933-z

Muratsubaki, H., Enomoto, K., Ichijoh, Y., and Yamamoto, Y. (2003). Hypertriglyceridemia associated with decreased post-heparin plasma hepatic triglyceride lipase activity in hypoxic rats. *Arch. Physiol. Biochem.* 111, 449–454. doi: 10.3109/13813450312331342319

Nakajima, K., Nakano, T., Tokita, Y., Nagamine, T., Inazu, A., Kobayashi, J., et al. (2011). Postprandial lipoprotein metabolism: VLDL vs chylomicrons. *Clin. Chim. Acta* 412, 1306–1318. doi: 10.1016/j.cca.2011.04.018

Nielsen, S., and Karpe, F. (2012). Determinants of VLDL-triglycerides production. *Curr. Opin. Lipidol.* 23, 321–326. doi: 10.1097/MOL.0b013e3283544956

Ntambi, J. M., and Miyazaki, M. (2004). Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog. Lipid Res.* 43, 91–104. doi: 10.1016/S0163-7827(03)00039-0

O'Rourke, R. W., Meyer, K. A., Gaston, G., White, A. E., Lumeng, C. N., and Marks, D. L. (2013). Hexosamine biosynthesis is a possible mechanism underlying hypoxia's effects on lipid metabolism in human adipocytes. *PLoS One* 8:e71165. doi: 10.1371/journal.pone.0071165

Packard, C. J., Boren, J., and Taskinen, M.-R. (2020). Causes and consequences of Hypertriglyceridemia. *Front. Endocrinol.* 11:252. doi: 10.3389/fendo.2020.00252

Raguso, C. A., Guinot, S. L., Janssens, J.-P., Kayser, B., and Pichard, C. (2004). Chronic hypoxia: common traits between chronic obstructive pulmonary disease and altitude. *Curr. Opin. Clin. Nutr. Metab. Care* 7, 411–417. doi: 10.1097/01.mco.0000134372.78438.09

Regazzetti, C., Peraldi, P., Grémeaux, T., Najem-Lendom, R., Ben-Sahra, I., Cormont, M., et al. (2009). Hypoxia decreases insulin signaling pathways in Adipocytes. *Diabetes Metab. Res. Rev.* 58, 95–103. doi: 10.2337/db08-0457

Savransky, V., Jun, J., Li, J., Nanayakkara, A., Fonti, S., Moser, A. B., et al. (2008). Dyslipidemia and atherosclerosis induced by chronic intermittent hypoxia are attenuated by deficiency of Stearoyl Coenzyme A Desaturase. *Circ. Res.* 103, 1173–1180. doi: 10.1161/CIRCRESAHA.108.178533

Savransky, V., Nanayakkara, A., Li, J., Bevans, S., Smith, P. L., Rodriguez, A., et al. (2007). Chronic intermittent hypoxia induces atherosclerosis. *Am. J. Respir. Crit. Care Med.* 175, 1290–1297. doi: 10.1164/rccm.200612-1771OC

Semenza, G. L. (2000). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol.* 88, 1474–1480. doi: 10.1152/jappl.2000.88.4.1474

Semenza, G. L. (2014). Hypoxia-inducible factor 1 and cardiovascular disease. *Annu. Rev. Physiol.* 76, 39–56. doi: 10.1146/annurev-physiol-021113-170322

Shimano, H. (2001). Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog. Lipid Res.* 40, 439–452. doi: 10.1016/S0163-7827(01)00010-8

Siques, P., Brito, J., Naveas, N., Pulido, R., De la Cruz, J. J., Mamani, M., et al. (2014). Plasma and liver lipid profiles in rats exposed to chronic hypobaric hypoxia: changes in metabolic pathways. *High Alt. Med. Biol.* 15, 388–395. doi: 10.1089/ham.2013.1134

Somers, V. K., Dyken, M. E., Clary, M. P., and Abboud, F. M. (1995). Sympathetic neural mechanisms in obstructive sleep apnea. *J. Clin. Invest.* 96, 1897–1904. doi: 10.1172/JCI118235

Somers, V. K., Mark, A. L., Zavala, D. C., and Abboud, F. M. (1989). Influence of ventilation and hypocapnia on sympathetic nerve responses to hypoxia in normal humans. *J. Appl. Physiol.* 67, 2095–2100. doi: 10.1152/jappl.1989.67.5.2095

van Marken Lichtenbelt, W. D., Schrauwen, P., van de Kerckhove, S., and Westerterp-Plantenga, M. S. (2002). Individual variation in body temperature and energy expenditure in response to mild cold. *Am. J. Physiol. Endocrinol. Metab.* 282, E1077–E1083. doi: 10.1152/ajpendo.00020.2001

Varela-Guruceaga, M., Milagro, F. I., Martínez, J. A., and de Miguel, C. (2018). Effect of hypoxia on caveolae-related protein expression and insulin signaling in adipocytes. *Mol. Cell. Endocrinol.* 473, 257–267. doi: 10.1016/j.mce.2018.01.026

Weiszenstein, M., Shimoda, L. A., Koc, M., Seda, O., and Polak, J. (2016). Inhibition of lipolysis ameliorates diabetic phenotype in a mouse model of obstructive Sleep Apnea. *Am. J. Respir. Cell Mol. Biol.* 55, 299–307. doi: 10.1165/rcmb.2015-0315OC

Westerterp, K. R., and Kayser, B. (2006). Body mass regulation at altitude. *Eur. J. Gastroenterol. Hepatol.* 18, 1–3. doi: 10.1097/00042737-200601000-00001

Wood, I. S., Stezhka, T., and Trayhurn, P. (2011). Modulation of adipokine production, glucose uptake and lactate release in human adipocytes by small changes in oxygen tension. *Pflügers Arch.* 462, 469–477. doi: 10.1007/s00424-011-0985-7

Wu, S. A., Kersten, S., and Qi, L. (2021). Lipoprotein lipase and its regulators: an unfolding story. *Trends Endocrinol. Metab.* 32, 48–61. doi: 10.1016/j.tem.2020.11.005

Xiao, C., Hsieh, J., Adeli, K., and Lewis, G. F. (2011). Gut-liver interaction in triglyceride-rich lipoprotein metabolism. *Am. J. Physiol. Endocrinol. Metab.* 301, E429–E446. doi: 10.1152/ajpendo.00178.2011

Yao, Q., Shin, M.-K., Jun, J. C., Hernandez, K. L., Aggarwal, N. R., Mock, J. R., et al. (2013). Effect of chronic intermittent hypoxia on triglyceride uptake in different tissues. *J. Lipid Res.* 54, 1058–1065. doi: 10.1194/jlr.M034272

Young, S. G., and Zechner, R. (2013). Biochemistry and pathophysiology of intravascular and intracellular lipolysis. *Genes Dev.* 27, 459–484. doi: 10.1101/gad.209296.112

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Urinary Metabolites as Predictors of Acute Mountain Sickness Severity

*Isaie Sibomana[1,2], Daniel P. Foose[3], Michael L. Raymer[3], Nicholas V. Reo[4], J. Philip Karl[5], Claire E. Berryman[5,6,7], Andrew J. Young[1,5,7], Stefan M. Pasiakos[5] and Camilla A. Mauzy[2]**

[1]Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, MD, United States, [2]Air Force Research Laboratory, 711th Human Performance Wing, Wright-Patterson AFB, Dayton, OH, United States, [3]Department of Computer Science and Engineering, Wright State University, Dayton, OH, United States, [4]Boonshoft School of Medicine, Wright State University, Dayton, OH, United States, [5]Military Nutrition Division, U.S. Army Research Institute of Environmental Medicine, Natick, MA, United States, [6]Department of Nutrition, Food, and Exercise Sciences, Florida State University, Tallahassee, FL, United States, [7]Oak Ridge Institute of Science and Education, Belcamp, MD, United States

Individuals sojourning at high altitude (≥2,500 m) often develop acute mountain sickness (AMS). However, substantial unexplained inter-individual variability in AMS severity exists. Untargeted metabolomics assays are increasingly used to identify novel biomarkers of susceptibility to illness, and to elucidate biological pathways linking environmental exposures to health outcomes. This study used untargeted nuclear magnetic resonance (NMR)-based metabolomics to identify urine metabolites associated with AMS severity during high altitude sojourn. Following a 21-day stay at sea level (SL; 55 m), 17 healthy males were transported to high altitude (HA; 4,300 m) for a 22-day sojourn. AMS symptoms measured twice daily during the first 5 days at HA were used to dichotomize participants according to AMS severity: moderate/severe AMS (AMS; $n = 11$) or no/mild AMS (NoAMS; $n = 6$). Urine samples collected on SL day 12 and HA days 1 and 18 were analyzed using proton NMR tools and the data were subjected to multivariate analyses. The SL urinary metabolite profiles were significantly different ($p \leq 0.05$) between AMS vs. NoAMS individuals prior to high altitude exposure. Differentially expressed metabolites included elevated levels of creatine and acetylcarnitine, and decreased levels of hypoxanthine and taurine in the AMS vs. NoAMS group. In addition, the levels of two amino acid derivatives (4-hydroxyphenylpyruvate and N-methylhistidine) and two unidentified metabolites (doublet peaks at 3.33 ppm and a singlet at 8.20 ppm) were significantly different between groups at SL. By HA day 18, the differences in urinary metabolites between AMS and NoAMS participants had largely resolved. Pathway analysis of these differentially expressed metabolites indicated that they directly or indirectly play a role in energy metabolism. These observations suggest that alterations in energy metabolism before high altitude exposure may contribute to AMS susceptibility at altitude. If validated in larger cohorts, these markers could inform development of a non-invasive assay to screen individuals for AMS susceptibility prior to high altitude sojourn.

Keywords: acute mountain sickness, susceptibility, altitude, hypoxia, NMR-based metabolomics, metabolite profiles, altitude sickness, urine

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Matiram Pun,
University of Calgary, Canada

Reviewed by:
Jan Stepanek,
Mayo Clinic Arizona, United States
Sanjeeb Sudarshan Bhandari,
Virginia Tech Carilion School of Medicine and Research Institute, United States

***Correspondence:**
Camilla A. Mauzy
camilla.mauzy@us.af.mil

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INTRODUCTION

At high altitude, hypobaric hypoxia elicits a series of physiological responses that are highly variable in humans. These responses assist in adapting to high altitude (HA; ≥2,500 m) conditions, but can also lead to development of acute mountain sickness (AMS) or life-threatening forms of altitude-induced illness such as high altitude cerebral edema (HACE) and high altitude pulmonary edema (HAPE; Luks et al., 2017). Non-acclimatized AMS-susceptible individuals usually develop AMS symptoms within 6–12 h after a rapid ascent and exposure to high altitudes (Bärtsch and Swenson, 2013) with symptoms generally resolving within 72 h of altitude exposure (TBMED505, 2010). While self-resolving, severe AMS symptoms can be temporarily debilitating. Such effects may be an unpleasant nuisance for leisure travelers but for military personnel, AMS can compromise occupational performance.

Currently, prevention of AMS onset involves pharmaceutical and non-pharmaceutical approaches. Pharmaceutical prophylaxis has limitations as medications such as acetazolamide (Dumont et al., 2000; Kayser et al., 2008; Low et al., 2012; Luks et al., 2014) are associated with side effects that, while mild, may discourage use. Non-pharmaceutical approaches include pre-acclimatization by intermittent exposure to normobaric hypoxia (Treml et al., 2020) or spending time at moderate altitude before ascending to higher elevations (Luks et al., 2017). While pre-acclimatization carries the benefit of reducing AMS, implementation can be logistically difficult. Identifying individuals at highest risk of severe AMS before ascent would be a useful decision aid for medical preparation and planning prior to high altitude sojourn.

Besides having a prior history of AMS occurrences, there are no clinical or routine laboratory examinations that can be performed to determine AMS susceptibility. As such, there is interest in developing rapid molecular-based screening methods for that purpose. For example, Canouï-Poitrine et al. (2014) developed and validated a model intended to identify individuals at risk of developing severe AMS and other forms of altitude-induced illness (HACE and HAPE). However, the model is not amenable to widespread and easy application, as it requires subjects to undergo an exercise test regimen, while breathing a hypoxic gas mixture. Moreover, the value and accuracy of that hypoxic cardiopulmonary exercise testing model has been questioned (Bärtsch, 2014). Yang et al. (2016) suggested that serum levels of three peptides (ITIH4 347–356, ITIH1 205–214, and FGA 588–624) at sea level can be used to determine the predisposition to high altitude-induced illness. However, the effectiveness and accuracy of these peptides in predicting AMS are yet to be established, and measuring these peptides requires invasive blood collection. An ideal screening platform would be non-invasive (e.g., urine) and easy to implement.

Genetic factors have been regarded as key players in high altitude adaptation (Arestegui et al., 2011; Yu et al., 2016; Bottura et al., 2019), suggesting that genetic polymorphisms influence high altitude adaptation (Yi et al., 2010). It is possible that functional polymorphisms in key enzymes involved in physiologic pathways may drive occurrence and severity of AMS and that metabolite outputs yielded by these pathways can be determined using a metabolomics-based approach.

Metabolomics is a unique top-down approach that can be applied to study complex systems (Nicholson and Lindon, 2008). The resultant metabolite profiles are regarded as good indicators of an organism's physiology as they measure the "end result" of multiple protein, gene, and environmental interactions (Wishart, 2007). As such, applying metabolomic approaches to examine physiological alterations resulting from altitude adaptation may not only identify biomarkers for AMS susceptibility, but may also provide further insight into the physiologic pathways affecting AMS. This exploratory effort used an untargeted metabolomics approach to identify urine metabolites that might serve as predictive markers of AMS susceptibility and provide insight into the biological pathways underpinning AMS.

MATERIALS AND METHODS

Subjects and Study Design

The analyses reported herein used archived samples and data from a study designed to assess the efficacy of a higher protein diet for preserving fat-free mass during high altitude (HA; 4,300 m) sojourn (Berryman et al., 2018). The present analyses were conceived after trial completion to explore whether measuring urine metabolites could provide insight into observed inter-individual variability in AMS severity (Karl et al., 2018a).

The study was approved by the Institutional Review Board at the United States Army Research Institute of Environmental Medicine (USARIEM) in Natick, MA and conducted May–Aug 2016. Investigators adhered to the policies for the protection of human participants as prescribed by Army Regulation 70–25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. The trial is registered on https://clinicaltrials.gov/, NCT02731066.

Seventeen healthy, unacclimatized, physically active men (aged 18–42 years) participated in the study. Although, study enrollment was open to both sexes, no women volunteered to participate. Study methods, and primary and secondary results have been previously reported in detail (Berryman et al., 2018; Karl et al., 2018a,b; Margolis et al., 2018; Pasiakos et al., 2018; Young et al., 2018; Hennigar et al., 2020). Briefly, the human study was a randomized, controlled trial consisting of two phases conducted over 43 consecutive days. During the 21-day first phase, participants resided at sea level (SL), consumed a self-selected weight maintaining diet, maintained habitual exercise routines, and were free living but visited the laboratory daily. On day 21, participants were flown from Boston, MA to Denver, CO, where they were placed on supplemental oxygen until arriving at the summit of Pike's Peak, CO (4,300 m) the following morning (day 0 at HA). Participants then resided for the next 21 days at the United States Army Research Institute of Environmental Medicine Maher Memorial Laboratory, Pike's Peak, CO (phase 2; HA). During HA, participants were under constant supervision, consumed a controlled and measured diet, and engaged in prescribed physical activity. Participants started consuming controlled diets on day 1, the first full day

of residence at 4,300 m, and continued until the end of the sojourn at HA (phase 2). Diets contained either a standard amount of protein (1.1 g/kg/day; $n=8$) or higher amount of protein (2.1 g/kg/day; $n=9$), and were designed to induce weight loss, which is common during military training and operations, and during HA sojourn (Hamad and Travis, 2006).

The prevalence and severity of AMS was assessed using the shortened version of the Environmental Symptoms Questionnaire (ESQ; Beidleman et al., 2007). The ESQ was administered twice daily during the first 5 days at HA and used to calculate AMS weighted cerebral factor scores (Beidleman et al., 2007). Peak scores were recorded from all participants during the first 48 h at HA, and were used to categorize AMS severity as mild ($\geq$0.7 and <1.53), moderate ($\geq$1.53 and <2.63), and severe ($\geq$2.63; Beidleman et al., 2013; Karl et al., 2018a). The analyses described below used two group identifiers: AMS and NoAMS. For the purposes of this analysis, subjects who scored less than 1.53 were designated as belonging to the NoAMS (no/mild AMS symptoms) group ($n=6$), while those with scores greater than 1.53 were designated as the AMS (moderate/severe AMS symptoms) group ($n=11$).

Urine Sample Collection and Preparation

Urine samples obtained at sea level 9 days prior to accent to altitude (SL) and at high altitude on days 1 (HA1) and 18 (HA2) were used for the analyses described herein. Collections began at 0730 on SL day 12 and 0700 on HA days 1 and 18 following an overnight fast and required participants to collect all urine produced over 2 h. During that 2-h period, participants consumed their individualized standard or higher protein breakfast on all 3 test days. Day 12 was the only day participants ate their diet group-specific breakfast during SL. Aliquots of the SL samples were frozen ^{1}H nuclear magnetic resonance (NMR) metabolomic analyses and shipped to Wright Patterson Air Force Base (WPAFB), Dayton, OH for analyses. Upon arrival at WPAFB, all urine samples were stored at −80°C. The preparation of urine samples for ^{1}H NMR spectral data acquisition followed the procedure described in Sibomana et al. (2017).

NMR Data Acquisition and Processing

All proton NMR spectra were acquired using a Varian INOVA NMR instrument operating at 600 MHz and a probe temperature of 25°C. NMR spectral data acquisition and processing are routinely performed in our laboratory. These procedures were conducted as previously described (Sibomana et al., 2017).

NMR Data Analyses

Multivariate data analyses were conducted on binned and scaled spectral data. Binned NMR data were scaled to the entire dataset chosen as reference by subtracting each bin value from the mean value for the corresponding bin in the reference data (whole dataset), then dividing this value by the SD of the reference data (auto-scaling).

Unsupervised Analysis

Principal Component Analysis (PCA) was used as an unsupervised analysis technique and provided a first approach for data visualization (Mahle et al., 2011). The PCA model was constructed based on the data for AMS group at SL and HA1. Data for the AMS group at HA2 and NoAMS group at SL, HA1, and HA2 were then superimposed onto the PCA scores plot. The quality of data clustering in this PCA model was evaluated using Davies-Bouldin (Davies and Bouldin, 1979) and Silhouette (Rousseeuw, 1987) indexes. Davies-Bouldin index (DBI) is defined as a ratio between the within group distances (intra group scatter) and the between group distances (group's separation). The lower the DBI value, the better the cluster separation and the tightness inside the groups. The silhouette value is a measure of how similar a data point is to its own cluster (group) compared to other clusters (groups). The higher the value, the better the data point matches to its own cluster (group) and the poorer it matches to neighboring clusters (groups).

Supervised Analysis

Orthogonal Projection onto Latent Structures – Discriminant Analysis (OPLS-DA) was used as a supervised technique to classify data and identify salient features that allow class separation of AMS vs. NoAMS at SL, HA1, and HA2 (Wold et al., 2001). The Q^2 (coefficient of prediction) metric was used to evaluate the predictive ability of the OPLS models as described in report of Sibomana et al. (2017). A permutation test was also performed to evaluate the significance of the Q^2 metric. This test involved repeatedly permuting the data labels and re-running the discrimination analysis, resulting in a distribution of the Q^2 scores (Westerhuis et al., 2008). The Q^2 from the correctly labeled data is then compared to the distribution to determine the significance of the model at a specified alpha (set herein as $\alpha=0.01$). A receiver operator characteristic (ROC) curve was also used as a secondary validation of an OPLS binary model and the area under the curve (AUC) was calculated. Evaluation of the significance of this AUC value was conducted using the same permutation procedure as described above.

The variable selection (salient bins) from OPLS-DA was statistically evaluated as described in Sibomana et al. (2017). Briefly, the bin loadings, commonly referred to as coefficients, were compared to calculated null distributions in order to select for significance. The null distribution for each bin was determined by refitting the OPLS model to datasets, in which each bin was independently and randomly permuted to remove any correlation between it and AMS/NoAMS groups. The true OPLS model loading was then compared to the resulting null distribution of loadings, and values in the tail (greater than 99.5% or less than 0.5% of the null distribution; corresponding to $\alpha=0.01$) were assumed to contribute significantly to the model. The permutation was initially repeated 1,000 times for each bin and those near-significant loadings (greater than 92.5% or less than 7.5% of the null distribution; corresponding to $\alpha=0.2$) were selected for 500 additional permutations (total 1,500).

Quantification of Metabolite Resonances

Normalized NMR spectra (PQN method; see above) were used to quantify metabolite resonances determined to be important

for group classification. Quantification of specific metabolite resonances was accomplished using an interactive spectral deconvolution algorithm in MATLAB adapted from Anderson et al. (2011). The deconvolution tool fits a defined spectral region using a combination of tunable baseline shapes (spline, v-shaped, linear, or constant) and a Gauss-Lorentz peak-fitting function. Metabolite peak intensities (total peak area) represent a semi-quantitative assessment of urine metabolites since this biofluid accumulates in the bladder over a variable period of time (i.e., 8 h) and its volume cannot be controlled. Although, the PQN method of spectral normalization helps to adjust for variable urine concentrations, absolute quantitative amounts of each metabolite are not reported. However, the semi-quantitative metabolite measurements reported herein do allow a relative comparison between samples.

Nuclear magnetic resonance spectral regions identified as significant by OPLS-DA were compared between time points (SL, HA1, and HA2) and AMS vs. NoAMS, and specific resonances were assigned to metabolites with the aid of literature, on-line databases (HMDB, http://www.hmdb.ca/, www.bmrb.wisc.edu, etc.), and by "spiking" samples with known compounds, if necessary. Signal intensities were integrated to obtain relative measures of metabolite concentrations at each time point.

Creatine Assays

Creatine assays were performed on additional archived urine samples collected at the same time as samples used for the NMR analysis. Assays were conducted using an Abcam (Cambridge, United Kingdom) creatine activity assay kit (ab65339) according to manufacturer instructions.

Statistical Analyses

A repeated measures MANOVA was conducted to examine effects of time and AMS status (AMS vs. NoAMS) on urine metabolite profiles. Only metabolites identified in OPLS-DA as significant were subjected to MANOVA. For metabolites demonstrating time-by-AMS group interactions ($p < 0.05$), Levene's and Welch's tests were conducted to assess the equality of variances between the data for SL, HA1, and HA2 or AMS vs. NoAMS groups for each metabolite using statistical software package JMP® 11.0.0 (SAS Institute, Cary, NC, United States). If Levene's test was significant ($p \leq 0.05$), then a Welch's nonparametric ANOVA test was used to determine if there were significant differences in the mean values between groups for the metabolite of interest. If the Levene's test was not significant, significance was tested using a one-way ANOVA (*t*-test). If both Levene's and Welch's tests were significant ($p \leq 0.05$), a pairwise Welch test was performed for all pairs of groups. No false discovery rate correction was applied to the data since OPLS-DA and MANOVA were used to down-select metabolites. Only metabolites identified by both data analysis methods were considered as statistically significant. Results are expressed as mean ± SEM and are considered statistically significant at $p \leq 0.05$. Cohen's d (effect size; Cohen, 1988) was used as a measure of the magnitude of changes in the level of each urinary metabolite noted at HA1 and HA2 relative to SL by subtracting the value obtained for SL from those obtained for HA (HA1 or HA2) and assessing the difference relative to the pooled SDs for HA (HA1 or HA2) and SL.

RESULTS

Urinary Metabolite Alterations Over Time

The mean peak AMS-weighted cerebral factor score for AMS individuals (2.25 ± 0.18; $n = 11$) was significantly elevated ($p < 0.05$) compared to in NoAMS subjects (0.78 ± 0.18; $n = 6$). AMS severity (i.e., NoAMS vs. AMS) was unrelated to diet group (Karl et al., 2018a). PCA analysis indicated that the urinary metabolite profiles for both groups changed over the time course of the study with the AMS group displaying greater variation in data at HA1 compared to NoAMS (**Figure 1**). PCA also clearly separated urinary profiles for AMS from NoAMS at all time points, with differences being most apparent at SL (**Figure 1**). The urinary profiles for both groups at HA2 indicated a trajectory returning toward SL. Mapping positions for NoAMS at SL and at HA2 partially overlapped, indicating some similarities in metabolite profiles. In contrast, there is a clear separation in profiles within the AMS group at these two time points.

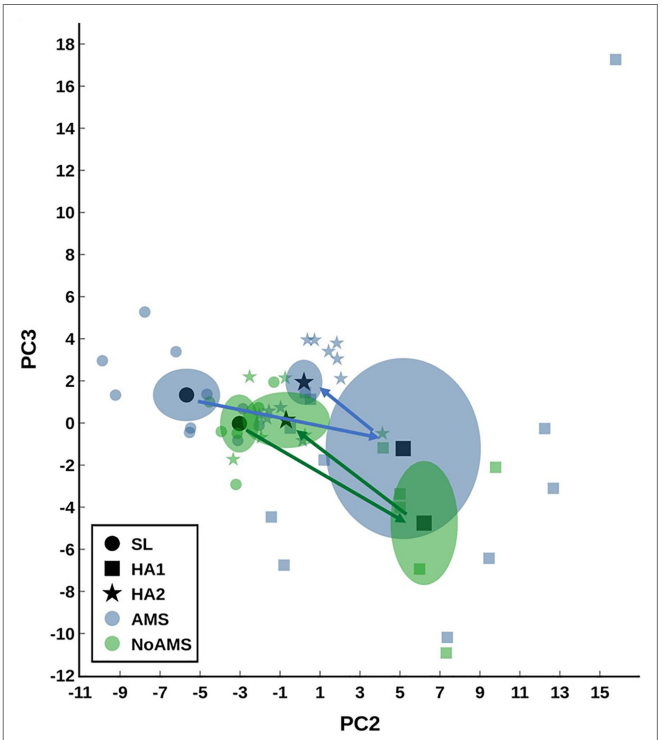

FIGURE 1 | Principal component analysis (PCA) scores plot modeling the urine data for acute mountain sickness (AMS) group at sea level (SL) and high altitude (HA) at Day 1 (HA1). Data for AMS group at high altitude day 18 (HA2) and those for no/mild AMS (NoAMS) group at SL, HA1, and HA2 are superimposed in the model. Data were autoscaled using all groups as reference. Data are plotted showing the centroid mean ± 2 SE (or 95% CI) as well as the individual data points. The arrows show the trajectory from SL to HA1 to HA2.

Changes in urinary metabolite levels that occurred from SL to HA1 and SL to HA2 indicated that changes in the levels of only four metabolites differed between AMS or No AMS groups (**Figure 2**). These metabolites included creatine (energy metabolism), two amino acid derivatives consisting of N-methylhistidine and acetylcarnitine, and hypoxanthine (nucleotide derivative). As shown in **Figure 2A**, urinary creatine levels decreased 64% from SL to HA1 in the AMS group, but increased by 256% in the NoAMS group. At HA2, creatine levels were 42% lower than that noted at SL in the AMS group, but increased by 190% from SL to HA2 in the NoAMS group. The levels of hypoxanthine (**Figure 2C**) and acetylcarnitine (**Figure 2D**) increased by 182 and 135%, respectively, from SL to HA1 for the AMS group, while in the NoAMS group, levels increased by 51 and 463%, respectively. At HA2, N-methylhistidine levels (**Figure 2B**) for AMS increased by 86% relative to SL, while NoAMS subjects decreased by 15%. It is noteworthy to indicate that creatine, hypoxanthine, and N-methylhistidine were among the metabolite classifiers of these two groups at SL.

Urinary Metabolites at Sea Level Compared to AMS Incidence

Orthogonal projection onto latent structures – discriminant analysis comparing AMS and NoAMS at SL (**Figure 3**) yielded a Q^2 value of 1.0 ($p = 0.001$), a predictive accuracy of 100% (leave-1-out cross validation) and an AUC value of 1.0 ($p = 0.001$). Further, the T-score scatter plots of the data confirmed that the urine metabolite profiles for the AMS group clustered together and were separated from the NoAMS group. Examination of metabolites that classified these two groups at SL indicated that creatine was the strongest driver of separation between the two groups (**Figure 4**). Spectra from the ^{1}H NMR analysis showed that AMS subjects had ($p \leq 0.05$) higher relative peak intensity for creatine at SL (1.34 ± 0.52) as compared to NoAMS subjects (0.11 ± 0.03; **Figure 4A**). These observations were confirmed by a secondary method using creatine assay analyses (**Figure 4B**). Additional metabolites driving discrimination between groups at SL included 4-hydroxyphenylpyruvate, taurine, N-methylhistidine, acetylcarnitine, hypoxanthine, and two unidentified metabolites (**Figure 5**). Urinary excretion levels

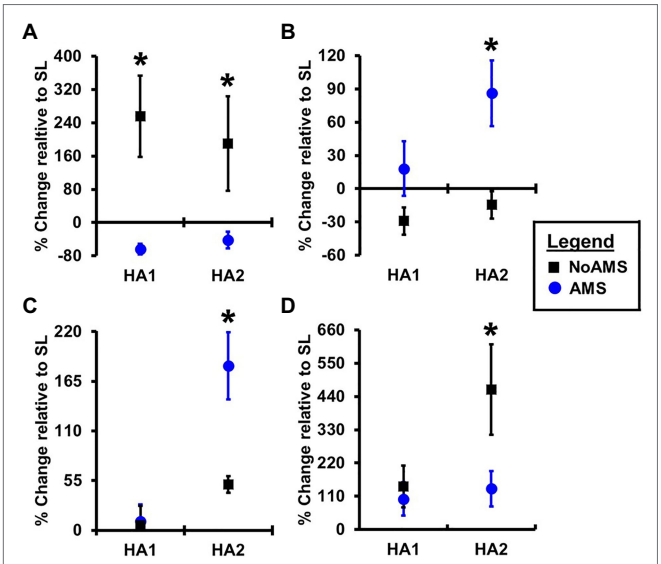

FIGURE 2 | Percent change in peak intensity of **(A)** creatine, **(B)** N-methylhistidine, **(C)** hypoxanthine, and **(D)** acetylcarnitine 4,300 m on day 1 (HA1) and day 18 (HA2) relative to SL for urine samples collected from AMS susceptible (blue symbol) and NoAMS resistant subjects (black symbol). Asterisk denotes significant difference between groups ($p \leq 0.05$).

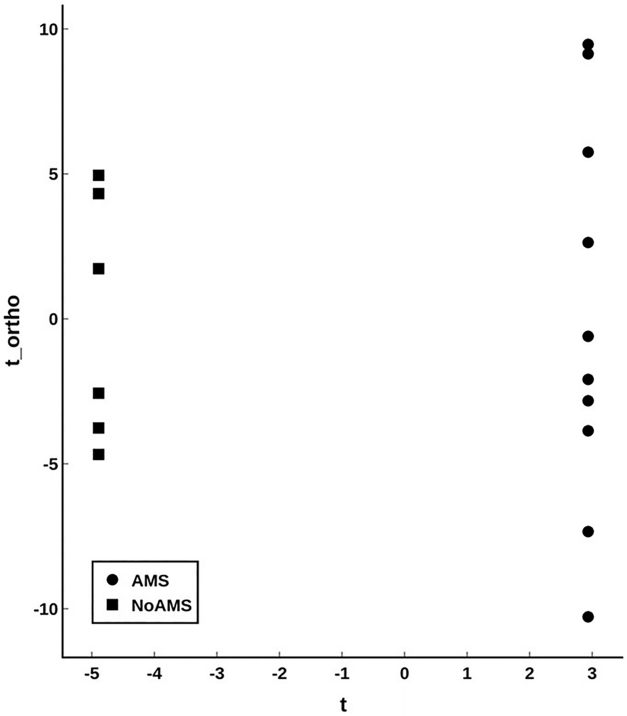

FIGURE 3 | Orthogonal projection onto latent structures – discriminant analysis (OPLS-DA; T-scores plot) modeling the urinary metabolite data for AMS (solid circle) vs. NoAMS (solid square) groups at SL. Data were autoscaled using all groups as reference. The analysis is highly significant with $Q^2 = 1.0$ ($p < 0.001$).

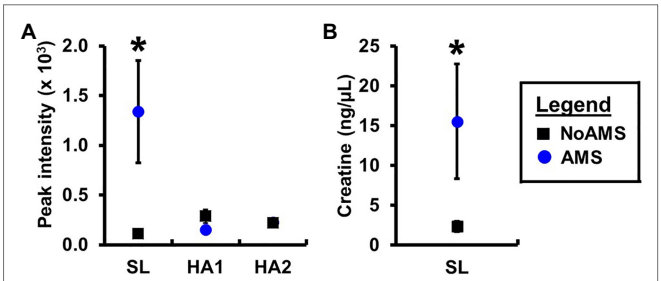

FIGURE 4 | Urinary creatine levels for AMS susceptible (blue symbol) and NoAMS resistant subjects (black symbol) at SL, and 4,300 m on day 1 (HA1) and day 18 (HA2) determined using the **(A)** ^{1}H NMR technique and **(B)** creatine assay. Asterisk denotes significant difference between groups ($p \leq 0.05$).

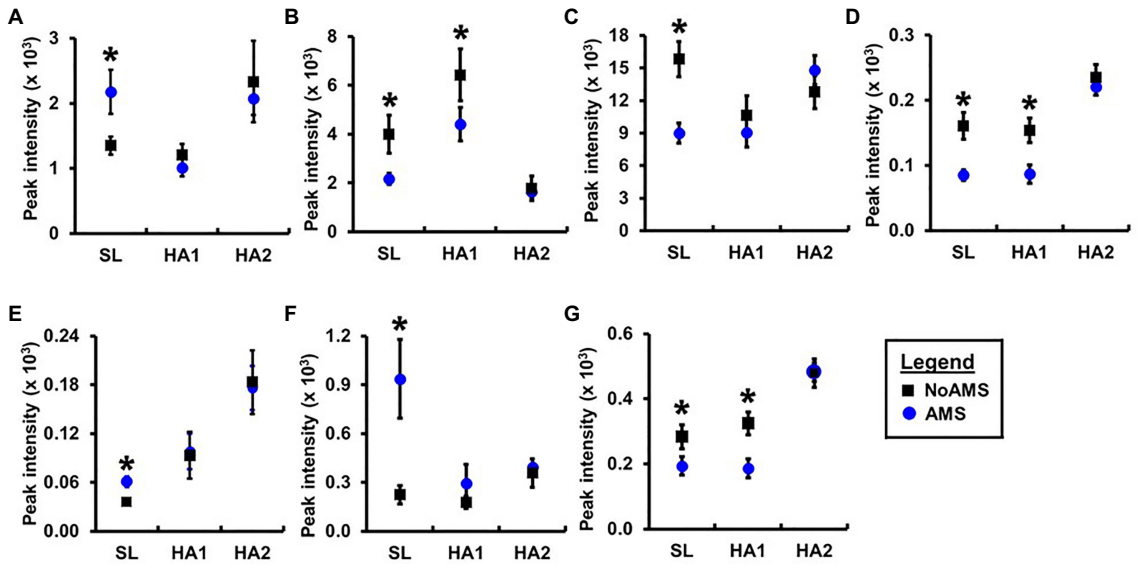

FIGURE 5 | Relative peak intensities of **(A)** 4-hydroxyphenylpyruvate, **(B)** taurine, **(C)** N-methylhistidine, **(D)** hypoxanthine, **(E)** acetylcarnitine, unidentified peaks at **(F)** 3.33 ppm (doublet) and **(G)** 8.20 ppm (singlet) in 1H nuclear magnetic resonance (NMR) spectral data for urine samples collected from AMS susceptible (blue symbol) and NoAMS resistant subjects (black symbol) at SL, and 4,300 m on day 1 (HA1) and day 18 (HA2). Asterisk denotes significant difference between groups ($p \leq 0.05$).

of taurine (**Figure 5B**), N-methylhistidine (**Figure 5C**), hypoxanthine (**Figure 5D**), and one unidentified metabolite (a singlet at 8.20 ppm; **Figure 5G**) were lower in AMS vs. NoAMS at SL, while the levels of 4-hydroxyphenylpyruvate (**Figure 5A**), acetylcarnitine (**Figure 5E**), and unidentified metabolite at 3.33 ppm (doublet; **Figure 5F**) were elevated in the AMS group. The levels of taurine, hypoxanthine, and unidentified metabolite at 8.20 ppm for AMS subjects were still lower than values obtained for the NoAMS group at HA1, but these differences between groups disappeared at HA2.

DISCUSSION

Changes in Urinary Metabolite Profiles Over Time

Principal component analysis results indicated that the urinary metabolite profiles for AMS and NoAMS groups changed significantly as the subjects moved from SL to HA and during their stay at altitude, reflecting the subject's response to altitude environment. The changes in metabolite profiles from SL to HA reflect alterations in metabolic pathways, which are likely driven by complex adaptive changes in multiple biological systems responding to hypobaric hypoxia. The AMS group displayed greater variation in data at HA1 (**Figure 1**) compared to NoAMS group, highlighting AMS subject's diverse responses to high altitude conditions. The observations that metabolite profiles for both groups were distinct at SL and became more similar at high altitude, suggest the existence of the urinary metabolite signatures for AMS susceptibility that may be apparent before exposure to altitude-induced stress. Thus, the discussion focuses on the metabolite differences between AMS and NoAMS at SL. Eight urinary metabolites that separated AMS from NoAMS individuals at sea level were identified. These metabolites included creatine, hypoxanthine, taurine, acetylcarnitine, N-methylhistidine, 4-HPPA, and two unknowns (**Figures 4, 5**).

Cellular Availability of Creatine and Hypoxantine in AMS Susceptible Subjects

Of the metabolite alterations seen at sea level, creatine had the highest contribution to the PCA segregation of NoAMS subjects. The average urinary creatine level in AMS susceptible individuals was 12-fold greater at sea level than NoAMS subjects (**Figure 4**). This difference could result from one or more factors including: (1) a higher dietary intake of creatine-containing foods, (2) a lower conversion rate of creatine to phosphocreatine and creatinine, and (3) decreased cellular retention of creatine. In this study, dietary protein intake did not differ between groups at sea level and volunteers reported compliance with instructions not to consume any supplements. While urinary phosphocreatine excretion was not examined, urine creatinine levels did not differ between groups. Therefore, we hypothesize that the simplest and most likely explanation for the higher creatine excretion rate in AMS individuals is decreased cellular retention.

Lower creatine cellular retention at sea level would lead to an increased rate of urinary elimination, limiting cellular availability of the substrate required for phosphocreatine synthesis once shifted to hypoxic conditions. The implication is that in AMS subjects, cells may have an existing deficiency in an energy supply needed to cope with altitude-induced hypoxia. Hypoxia is known to affect cellular ATP production through

downregulation of several tricarboxylic cycle enzymes (Green et al., 1989; Howald et al., 1990; Levett et al., 2012, 2015; Murray and Horscroft, 2016) as well as compromising electron transport chain complexes (Howald et al., 1990; Levett et al., 2012, 2015; Colleoni et al., 2013; Murray and Horscroft, 2016). Indeed, several studies also suggest that lower cellular creatine levels increase sensitivity to hypoxia (Wilken et al., 1996; Turner et al., 2015; Scheer et al., 2016). In contrast, Turner et al. (2015) reported that hypoxia-induced decrements in a wide range of neuropsychological measures were corrected by creatine supplementation. Other studies suggest that stored phosphocreatine may play a significant role in sustaining synaptic transmission during hypoxia (Lipton and Whittingham, 1982), and that creatine supplementation can enhance the cellular adaptive response to hypoxia mediated by HIF-1 in cardiomyocytes (Santacruz et al., 2017). Collectively, these findings suggest that cellular creatine availability is critical to sustaining intracellular phosphocreatine and ATP pools during hypoxic conditions. Our findings of increased urinary excretion of creatine at sea level in AMS subjects suggest that existing deficiencies of cellular creatine levels may increase hypoxia sensitivity.

Hypoxanthine was also among the metabolites that classified AMS and NoAMS groups at SL (**Figure 5D**). Hypoxanthine is a naturally occurring purine degradation by-product, and cellular levels are associated with cellular levels of creatine. For example, hypoxanthine supplementation has been shown to reverse hypoxia-induced depletion of cellular creatine and phosphocreatine pools (Lee et al., 2018). Findings of the present study suggest that cellular levels of hypoxanthine may be lower in AMS subjects which could, in turn, impair the cellular retention of creatine and account for its higher urinary excretion. While a correlation analysis using the ^{1}H NMR urinary creatine and hypoxanthine data did not indicate a creatine/hypoxanthine correlation for NoAMS individuals (**Figures 6A–C**), data for AMS individuals demonstrated a positive relationship between these two metabolites at SL ($R^2 = 0.4309$; **Figure 6D**) but not at HA1 (**Figure 6E**) or HA2 (**Figure 6F**).

Other Urinary Metabolite Differences Seen in Acute Mountain Sickness Susceptibility

Acute mountain sickness subjects also demonstrated significantly lower taurine excretion at sea level and Day 1 at altitude relative to NoAMS individuals. Of note, previous studies have suggested that this biogenic amine plays a significant role in protecting cells against hypoxia-induced damage (Crass and Lombardini, 1977; Franconi et al., 1985; Malcangio et al., 1989; Michalk et al., 1997; Amano et al., 2003; Chen et al., 2009, 2013). Further, under hypoxic conditions, taurine supplementation has been shown to improve cardiovascular function in pigs (Franconi et al., 1985), attenuate vascular remodeling in rats (Amano et al., 2003), and prevent learning impairment and increase survival time in mice (Malcangio et al., 1989). Although, taurine's mechanisms of protection against hypoxia-mediated decrements are not well understood, taurine may act as a potent endogenous agent to induce cellular growth despite oxygen deficiency, and improve both osmotic status and calcium homeostasis (Michalk et al., 1997). Collectively, these findings suggest that taurine may play an important role in counteracting hypoxic-induced cellular damage. The lower urinary excretion of taurine seen at sea level and Day 1 at altitude in AMS subjects may reflect an increase in degradation of this metabolite. Unfortunately, the current study did not investigate the taurine catabolism pathway.

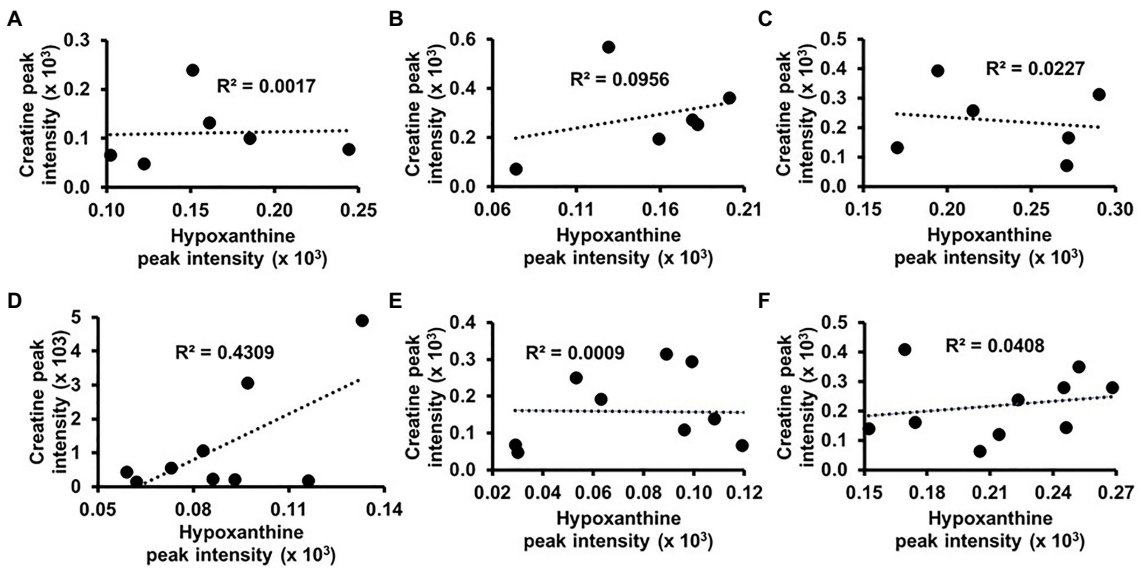

FIGURE 6 | Correlation analyses between urinary levels of creatine and hypoxanthine for NoAMS subjects at **(A)** SL, **(B)** 4,300 m on day 1 (HA1), **(C)** 4,300 m on day 18 (HA2), and AMS-susceptible individuals at **(D)** SL, **(E)** HA1, and **(F)** HA2.

Acetylcarnitine plays a critical role in cellular energy metabolism and has been shown to play a role in cellular responses to hypoxia-induced stress (Aureli et al., 1994; Scafidi et al., 2010). Barhwal et al. (2007) demonstrated that daily supplementation of acetylcarnitine to rats during hypoxic exposure ameliorated hypoxia-induced deficits in spatial working memory, oxidative stress, and apoptotic cascades, suggesting that this metabolite plays a significant role in the body's response to hypoxic stress. In the current study, urinary acetylcarnitine excretion in AMS susceptible individuals was higher than for NoAMS individuals at SL (**Figure 5E**). This may suggest that the cellular stores of this metabolite were lower in AMS individuals, and their increased susceptibility to AMS may be mediated by alteration in energy or lipid metabolism.

Urinary N-methylhistidine is formed in the body through methylation of peptide-bound histidine in muscle actin and myosin and eliminated in urine after protein breakdown (Long et al., 1975). Urinary excretion of N-methylhistidine is regarded as useful indicator for muscle protein breakdown provided that the individual has a meat-free diet (Munro and Young, 1978; Tomas et al., 1979; Elia et al., 1980, 1981). Though dietary protein can affect urinary excretion (Omstedt et al., 1978), it is unlikely in this study that the diet was driving the lower N-methylhistidine in AMS vs. NoAMS as dietary protein intake did not differ at SL. Of note, previous studies have shown that the levels of N-methylhistidine are altered in individuals sensitive to high altitude. For example, plasma levels of methylhistidine have previously been shown to increase in patients with HAPE compared to controls (Luo et al., 2012). That report conflicts with our findings on urinary levels of N-methylhistidine. However, it is possible that the disparity between the two studies derives from differences in the test matrices (i.e., urine vs. blood) used.

Finally, increased urinary excretion at sea level of 4-HPPA in AMS individuals suggest a pre-existing alteration in the phenylalanine catabolism pathway and/or 4-HPPA degradation pathway may contribute to AMS susceptibility. However, phenylalanine and tyrosine levels in the urine were not statistically different between groups. As the downstream of 4-HPPA degradation pathway was not investigated in this study, a more thorough study examining the molecular mechanisms for excessive 4-HPPA urinary elimination awaits future efforts.

Study Limitations and Future Study Modifications

In the current study, the diet was not a controlled variable at sea level (except on day 12) and day 0 at high altitude prior to starting the study diet regimen. As such, further studies are needed to investigate whether potential dietary factors can exert significant impact on the individual's susceptibility or resistance to AMS and should include a control group with controlled dietary input throughout the study, at sea level as well as at altitude. In addition, the sample size was limited to $n = 11$ and $n = 6$ for AMS and NoAMS groups, respectively. Follow-up studies using a larger sample sizes will be required to validate the biomarker potential as described here. In addition, the subjects spent 21 days at SL and 22 days at HA with sample collection limited to one time at SL (Day 12) and two times at altitude (Day 1 and Day 18). Since there was a considerable amount of time elapsing between sample collections at altitude, the sampling scheme did not capture temporal changes that may have transpired, especially during the 0–72 h period at altitude when the greatest molecular changes are expected to occur. Future studies should also consider additional sample collection time points at SL to establish a firm and consistent baseline. Lastly, female subjects should also be included in future efforts to allow examination of gender-related responsivity and, possibly, unique gender-based metabolite signatures.

CONCLUSION

This study identified a set of eight urinary metabolites using NMR-based metabolomics that, at sea level prior to altitude exposures, discriminated individuals who later experienced more severe acute mountain sickness upon ascent to high altitude. Urinary creatine, hypoxanthine, acetylcarnitine, 4-HPPA, N-methylhistidine, and taurine were among the classifiers of acute mountain sickness sensitive individuals. The observed metabolite differences between AMS and NoAMS at sea level reflect modulations in metabolic pathways that may result from genetic differences and other interacting factors. However, an examination of literature suggests that the urinary levels of these metabolites, directly or indirectly, play a role in energy metabolism and have been shown in other studies to influence physiologic responses to hypoxia in *in vivo* and *in vitro* models.

These results suggest that a specific set of urinary metabolites could potentially be used to identify AMS susceptible subjects even before exposure to altitude and exhibition of altitude-mediated sickness symptoms. If biological plausibility can be confirmed and findings validated in larger cohorts, these metabolites may comprise a metabolic biomarker signature that can potentially be used to non-invasively screen individuals for vulnerability to altitude-induced illness. In addition to biomarker development, these metabolites may provide insight into specific mechanisms involved in the pathophysiological process of AMS. This information would be of importance in designing new individualized approaches and therapeutics that can prevent or attenuate the impact of AMS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (USARIEM) in

Natick, MA. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IS: metabolomics methodology, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, and visualization. DF and MR: metabolomics formal analysis. NR: metabolomics formal analysis and writing – review and editing. JK: human study conceptualization, methodology, formal analysis, investigation, resources, data curation, writing – review and editing, visualization, supervision, and project administration. CB: human study conceptualization and analysis, methodology, formal analysis, investigation, data curation, writing – review and editing, and project administration. AY: human study conceptualization, methodology, investigation, supervision, and writing – review and editing. SP: human study conceptualization, methodology, resources, investigation, supervision, and writing – review and editing. CM: metabolomics formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualization, supervision, and project management. All authors contributed to the article and approved the submitted version.

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REFERENCES

Amano, H., Maruyama, K., Naka, M., and Tanaka, T. (2003). Target validation in hypoxia-induced vascular remodeling using transcriptome/metabolome analysis. *Pharm. J.* 3, 183–188. doi: 10.1038/sj.tpj.6500177

Anderson, P. E., Mahle, D. A., Doom, T. E., Reo, N. V., Del Raso, N. J., and Raymer, M. L. (2011). Dynamic adaptive binning: an improved quantification technique for NMR spectroscopic data. *Metabolomics* 7, 179–190. doi: 10.1007/s11306-010-0242-7

Arestegui, A. H., Fuquay, R., Sirota, J., Swenson, E. R., Schoene, R. B., Jefferson, J. A., et al. (2011). High altitude renal syndrome (HARS). *J. Am. Soc. Nephrol.* 22, 1963–1968. doi: 10.1681/ASN.2010121316

Aureli, T., Miccheli, A., Di Cocco, M. E., Ghirardi, O., Giuliani, A., Ramacci, M. T., et al. (1994). Effect of acetyl-L-carnitine on recovery of brain phosphorus metabolites and lactic acid level during reperfusion after cerebral ischemia in the rat--study by 13P- and 1H–NMR spectroscopy. *Brain Res.* 643, 92–99. doi: 10.1016/0006-8993(94)90013-2

Barhwal, K., Singh, S. B., Hota, S. K., Jayalakshmi, K., and Ilavazhagan, G. (2007). Acetyl-L-carnitine ameliorates hypobaric hypoxic impairment and spatial memory deficits in rats. *Eur. J. Pharmacol.* 570, 97–107. doi: 10.1016/j.ejphar.2007.05.063

Bärtsch, P. (2014). Con: hypoxic cardiopulmonary exercise testing identifies subjects at risk for severe high altitude illnesses. *High Alt. Med. Biol.* 15, 318–320. doi: 10.1089/ham.2013.1145

Bärtsch, P., and Swenson, E. R. (2013). Clinical practice: acute high-altitude illnesses. *N. Engl. J. Med.* 368, 2294–2302. doi: 10.1056/NEJMcp1214870

Beidleman, B. A., Muza, S. R., Fulco, C. S., Rock, P. B., and Cymerman, A. (2007). Validation of a shortened electronic version of the environmental symptoms questionnaire. *High Alt. Med. Biol.* 8, 192–199. doi: 10.1089/ham.2007.1016

Beidleman, B. A., Tighiouart, H., Schmid, C. H., Fulco, C. S., and Muza, S. R. (2013). Predictive models of acute mountain sickness after rapid ascent to various altitudes. *Med. Sci. Sports Exerc.* 45, 792–800. doi: 10.1249/MSS.0b013e31827989ec

Berryman, C. E., Young, A. J., Karl, J. P., Kenefick, R. W., Margolis, L. M., Cole, R. E., et al. (2018). Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. *FASEB J.* 32, 894–905. doi: 10.1096/fj.201700915R

Bottura, R. M., Lima, G. H. O., Hipolide, D. C., and Pesquero, J. B. (2019). Association between ACTN3 and acute mountain sickness. *Genes Environ.* 41:18. doi: 10.1186/s41021-019-0133-8

Canouï-Poitrine, F., Veerabudun, K., Larmignat, P., Letounel, M., Bastuji-Garin, S., and Richalet, J. P. (2014). Risk prediction score for severe high altitude illness: a cohort study. *PLoS One* 9:e100642. doi: 10.1371/journal.pone.0100642

Chen, P. C., Pan, P., Gharibani, P. M., Prentice, H., and Wu, J. Y. (2013). Taurine exerts robust protection against hypoxia and oxygen/glucose deprivation in human neuroblastoma cell culture. *Adv. Exp. Med. Biol.* 775, 167–175. doi: 10.1007/978-1-4614-6130-2_14

Chen, K., Zhang, Q., Wang, J., Liu, F., Mi, M., Xu, H., et al. (2009). Taurine protects transformed rat retinal ganglion cells from hypoxia-induced apoptosis by preventing mitochondrial dysfunction. *Brain Res.* 1279, 131–138. doi: 10.1016/j.brainres.2009.04.054

Cohen, J. (1988). *Statistical Power Analysis for the Behavioral Sciences*. *2nd Edn.* Mahwah, NJ: Lawrence Erlbaum Associates.

Colleoni, F., Padmanabhan, N., Yung, H. W., Watson, E. D., Cetin, I., Tissot van Patot, M. C., et al. (2013). Suppression of mitochondrial electron transport chain function in the hypoxic human placenta: a role for miRNA-210 and protein synthesis inhibition. *PLoS One* 8:e55194. doi: 10.1371/journal.pone.0055194

Crass, M. III, and Lombardini, J. B. (1977). Loss of cardiac muscle taurine after acute left ventricular ischemia. *Life Sci.* 21, 951–958. doi: 10.1016/0024-3205(77)90261-2

Davies, D. L., and Bouldin, D. W. (1979). A cluster separation measure. *IEEE Trans. Pattern Anal. Mach. Intell.* PAMI-1, 224–227. doi: 10.1109/TPAMI.1979.4766909

Dumont, L., Mardirosoff, C., and Tramèr, M. R. (2000). Efficacy and harm of pharmacological prevention of acute mountain sickness: quantitative systematic review. *BMJ* 321, 267–272. doi: 10.1136/bmj.321.7256.267

Elia, M., Carter, A., Bacon, S., and Smith, R. (1980). The effect of 3-methylhistidine in food on its urinary excretion in man. *Clin. Sci.* 59, 509–511. doi: 10.1042/cs0590509

Elia, M., Carter, A., Bacon, S., Winearls, C. G., and Smith, R. (1981). Clinical usefulness of urinary 3-methylhistidine excretion in indicating muscle protein breakdown. *Br. Med. J.* 282, 351–354. doi: 10.1136/bmj.282.6261.351

Franconi, F., Stendardi, I., Failli, P., Matucci, R., Baccaro, C., Montorsi, L., et al. (1985). The protective effects of taurine on hypoxia (performed in the absence of glucose) and on reoxygenation (in the presence of glucose) in Guinea-pig heart. *Biochem. Pharmacol.* 34, 2611–2615. doi: 10.1016/0006-2952(85)90556-8

Green, H. J., Sutton, J. R., Cymerman, A., Young, P. M., and Houston, C. S. (1989). Operation Everest II: adaptations in human skeletal muscle. *J. Appl. Physiol.* 66, 2454–2461. doi: 10.1152/jappl.1989.66.5.2454

Hamad, N., and Travis, S. P. (2006). Weight loss at high altitude: pathophysiology and practical implications. *Eur. J. Gastroenterol. Hepatol.* 18, 5–10. doi: 10.1097/00042737-200601000-00002

Hennigar, S. R., Berryman, C. E., Kelley, A. M., Anderson, B. J., Young, A. J., McClung, J. P., et al. (2020). High-altitude acclimatization suppresses hepcidin expression during severe energy deficit. *High Alt. Med. Biol.* 21, 232–236. doi: 10.1089/ham.2019.0109

Howald, H., Pette, D., Simoneau, J. A., Uber, A., Hoppeler, H., and Cerretelli, P. (1990). Effect of chronic hypoxia on muscle enzyme activities. *Int. J. Sports Med.* 11, S10–S14. doi: 10.1055/s-2007-1024847

Karl, J. P., Berryman, C. E., Young, A. J., Radcliffe, P. N., Branck, T. A., Pantoja-Feliciano, I. G., et al. (2018a). Associations between the gut microbiota and host responses to high altitude. *Am. J. Physiol. Gastrointest. Liver Physiol.* 315, G1003–G1015. doi: 10.1152/ajpgi.00253.2018

Karl, J. P., Cole, R. E., Berryman, C. E., Finlayson, G., Radcliffe, P. N., Kominsky, M. T., et al. (2018b). Appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at high atitude, but are not affected by protein intake. *High Alt. Med. Biol.* 19, 156–169. doi: 10.1089/ham.2017.0155

Kayser, B., Hulsebosch, R., and Bosch, F. (2008). Low-dose acetylsalicylic acid analog and acetazolamide for prevention of acute mountain sickness. *High Alt. Med. Biol.* 9, 13–23. doi: 10.1089/ham.2007.1037

Lee, J. S., Wang, R. X., Alexeev, E. E., Lanis, J. M., Battista, K. D., Glover, L. E., et al. (2018). Hypoxanthine is a checkpoint stress metabolite in colonic epithelial energy modulation and barrier function. *J. Biol. Chem.* 293, 6039–6051. doi: 10.1074/jbc.RA117.000269

Levett, D. Z., Radford, E. J., Menassa, D. A., Graber, E. F., Morash, A. J., Hoppeler, H., et al. (2012). Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *FASEB J.* 26, 1431–1441. doi: 10.1096/fj.11-197772

Levett, D. Z., Vigano, A., Capitanio, D., Vasso, M., De Palma, S., Moriggi, M., et al. (2015). Changes in muscle proteomics in the course of the Caudwell research expedition to Mt. Everest. *Proteomics* 15, 160–171. doi: 10.1002/pmic.201400306

Lipton, P., and Whittingham, T. S. (1982). Reduced ATP concentration as a basis for synaptic transmission failure during hypoxia in the in vitro Guinea-pig hippocampus. *J. Physiol.* 325, 51–65. doi: 10.1113/jphysiol.1982.sp014135

Long, C. L., Haverberg, L. N., Young, V. R., Kinney, J. M., Munro, H. N., and Geiger, J. W. (1975). Metabolism of 3-methylhistidine in man. *Metabolism* 24, 929–935. doi: 10.1016/0026-0495(75)90084-0

Low, E. V., Avery, A. J., Gupta, V., Schedlbauer, A., and Grocott, M. P. W. (2012). Identifying the lowest effective dose of acetazolamide for the prophylaxis of acute mountain sickness: systematic review and meta-analysis. *BMJ* 345:e6779. doi: 10.1136/bmj.e6779

Luks, A., McIntosh, S. E., Grissom, C. K., Auerbach, P. S., Rodway, G. W., Schoene, R. B., et al. (2014). Wilderness medical society practice guidelines for the prevention and treatment of acute altitude illness: 2014 update. *Wilderness Environ. Med.* 25, S4–S14. doi: 10.1016/j.wem.2014.06.017

Luks, A. M., Swenson, E. R., and Bärtsch, P. (2017). Acute high-altitude sickness. *Eur. Respir. Rev.* 26:160096. doi: 10.1138/16000617.0096-2016

Luo, Y., Zhu, J., and Gao, Y. (2012). Metabolomic analysis of the plasma of patients with high-altitude pulmonary edema (HAPE) using 1H NMR. *Mol. BioSyst.* 8, 1783–1788. doi: 10.1039/c2mb25044f

Mahle, D. A., Anderson, P. E., Del Raso, N. J., Raymer, M. L., Neuforth, A. E., and Reo, N. V. (2011). A generalized model for metabolomic analyses: application to dose and time dependent toxicity. *Metabolomics* 7, 206–216. doi: 10.1007/s11306-010-0246-3

Malcangio, M., Bartolini, A., Ghelardini, C., Bennardini, F., Malmberg-Aiello, P., Franconi, F., et al. (1989). Effect of ICV taurine on the impairmentof learning convulsions and death caused by hypoxia. *Psychopharmacology* 98, 316–320. doi: 10.1007/BF00451681

Margolis, L. M., Carbone, J. W., Berryman, C. E., Carrigan, C. T., Murphy, N. E., Ferrando, A. A., et al. (2018). Severe energy deficit at high altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity. *FASEB J.* 32, 5955–5966. doi: 10.1096/fj.201800163RR

Michalk, D. V., Wingenfeld, P., and Licht, C. (1997). Protection against cell damage due to hypoxia and reoxygenation: the role of taurine and the involved mechanisms. *Amino Acids* 13, 337–346. doi: 10.1007/BF01372597

Munro, H. N., and Young, V. R. (1978). Urinary excretion of N-methylhistidine (3-methylhistidine): a tool to study metabolic responses in relation to nutrient and hormonal status in health and disease of man. *Am. J. Clin. Nutr.* 31, 1608–1614. doi: 10.1093/ajcn/31.9.1608

Murray, A. J., and Horscroft, J. A. (2016). Mitochondrial function at extreme high altitude. *J. Physiol.* 594, 1137–1147. doi: 10.1113/JP270079

Nicholson, J. K., and Lindon, J. C. (2008). Systems biology: metabonomics. *Nature* 455, 1054–1056. doi: 10.1038/4551054a

Omstedt, P. T., Kihlberg, R., Tingvall, P., and Shenkin, A. (1978). Effect of dietary protein on urinary excretion of 3-methylhistidine in rat. *J. Nutr.* 108, 1877–1883. doi: 10.1093/jn/108.12.1877

Pasiakos, S. M., Berryman, C. E., Carbone, J. W., Murphy, N. E., Carrigan, C. T., Bamman, M. M., et al. (2018). Muscle Fn14 gene expression is associated with fat-free mass retention during energy deficit at high altitude. *Phys. Rep.* 6:e13801. doi: 10.14814/phy2.13801

Rousseeuw, P. (1987). Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. *J. Comput. Appl. Math.* 20, 53–65. doi: 10.1016/0377-0427(87)90125-7

Santacruz, L., Arciniegas, A. J. L., Darrabie, M., Mantilla, J. G., Baron, R. M., Bowles, D. E., et al. (2017). Hypoxia decreases creatine uptake in cardiomyocytes, while creatine supplementation enhances HIF activation. *Phys. Rep.* 5:e13382. doi: 10.14814/phy2.13382

Scafidi, S., Fiskum, G., Lindauer, S. L., Bamford, P., Shi, D., Hopkins, I., et al. (2010). Metabolism of acetyl-L-carnitine for energy and neurotransmitter synthesis in the immature rat brain. *J. Neurochem.* 114, 820–831. doi: 10.1111/j.1471-4159.2010.06807.x

Scheer, M., Bischoff, A. M., Kruzliak, P., Opatrilova, R., Bovell, D., and Büsselberg, D. (2016). Creatine and creatine pyruvate reduce hypoxia-induced effects on phrenic nerve activity in the juvenile mouse respiratory system. *Exp. Mol. Pathol.* 101, 157–162. doi: 10.1016/j.yexmp.2016.07.006

Sibomana, I., Del Raso, N. J., Mattie, D., Raymer, R., and Reo, N. (2017). Furosemide enhances the sensitivity of urinary metabolomics for assessment of kidney function. *Metabolomics* 13:24. doi: 10.1007/s11306-017-1162-6

TBMED505 (2010). "Altitude acclimatization and illness management," in *Technical Bulletin*. ed. Office of the Surgeon General (Headquarters, Department of the Army), 128.

Tomas, F. M., Ballard, F. J., and Pope, L. M. (1979). Age-dependent changes in the rate of myofibrillar protein degradation in humans as assessed by 3-methylhistidine and creatinine excretion. *Clin. Sci.* 56, 341–346. doi: 10.1042/cs0560341

Treml, T., Kleinsasser, A., Hell, T., Knotzer, H., Wille, M., and Burtcher, M. (2020). Carry-over quality of pre-acclimatization to altitude elicited by intermittent hypoxia: a participant-blinded, randomized controlled trial on antedated acclimatization to altitude. *Front. Physiol.* 11:531. doi: 10.3389/fphys.2020.00531

Turner, C. E., Byblow, W. D., and Gant, N. (2015). Creatine supplementation enhances corticomotor excitability and cognitive performance during oxygen deprivation. *J. Neurosci.* 35, 1773–1780. doi: 10.1523/JNEUROSCI.3113-14.2015

Westerhuis, J., Hoefsloot, H., Smit, S., Vis, D. J., Smilde, A. K., van Velzen, A. J. J., et al. (2008). Assessment of PLSDA cross validation. *Metabolomics* 4, 81–89. doi: 10.1007/s11306-007-0099-6

Wilken, B., Ramirez, J. M., Richter, D. W., and Hanefeld, F. (1996). The response to hypoxia is affected by creatine in the central respiratory network of mammals 251. *Pediatr. Res.* 40:557. doi: 10.1203/00006450-199609000-00274

Wishart, D. (2007). "Metabolomics in humans and other mammals," in *Metabolome Analysis: An Introduction*. eds. S. G. Villas-Boas, U. Roessner, M. A. E. Hansen, J. Smedsgaard and J. Nielsen (Hoboken, NJ: John Wiley & Sons, Inc.), 253–288.

Wold, S., Sjostrom, M., and Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemom. Intell. Lab. Syst.* 58, 109–130. doi: 10.1016/S0169-7439(01)00155-1

Yang, J., Li, W., Liu, S., Yuan, D., Guo, Y., Jia, C., et al. (2016). Identification of novel serum peptide biomarkers for high-altitude adaptation: a comparative approach. *Sci. Rep.* 6:25489. doi: 10.1038/s41598-016-0027-y

Yi, X., Liang, Y., Huerta-Sanchez, E., Jin, X., Cuo, Z. X. P., Pool, J. E., et al. (2010). Sequencing of 50 human exomes reveals adaptation to high altitude. *Science* 329, 75–78. doi: 10.1126/science.1190371

Young, A. J., Berryman, C. E., Kenefick, R. W., Derosier, A. N., Margolis, L. M., Wilson, M. A., et al. (2018). Altitude acclimatization alleviates the hypoxia-induced suppression of exogenous glucose oxidation during steady-state aerobic exercise. *Front. Physiol.* 9:830. doi: 10.3389/fphys.2018.00830

Yu, J., Zeng, Y., Chen, G., Bian, S., Qiu, Y., Liu, X., et al. (2016). Analysis of high-altitude syndrome and the underlying gene polymorphisms associated with acute mountain sickness after a rapid ascent to high-altitude. *Sci. Rep.* 6:38323. doi: 10.1038/srep38323

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Spleen Contraction During Sudden Eupneic Hypoxia Elevates Hemoglobin Concentration

Frank Pernett[1], *Felix Schagatay*[2], *Caroline Vildevi*[3] *and Erika Schagatay*[1,3]*

[1] Department of Health Sciences, Mid Sweden University, Östersund, Sweden, [2] Scandinavian Aviation Academy, Västerås, Sweden, [3] Swedish Winter Sports Research Centre, Östersund, Sweden

The spleen contracts progressively during moderate normobaric hypoxia exposure of 20 min, which elevates hemoglobin concentration (Hb). However, acute hypoxia exposure could be shorter and more severe when oxygen systems fail during, e.g., high-altitude sky diving, aircraft cabin pressure drop, balloon flights, extreme altitude climbing, and in some maladies. We aimed to evaluate the speed and magnitude of spleen contraction during short exposure to extreme eupneic hypoxia and its subsequent recovery on oxygen. Eight female and seven male volunteers were exposed to normobaric hypoxia (10% oxygen) for 10 min during sitting rest, followed by 10 min on 100% oxygen. Heart rate (HR), arterial oxygen saturation (SpO_2), and mean arterial blood pressure (MAP) were measured continuously. The spleen was measured via ultrasonic imaging every minute for volume calculations, and venous blood samples were drawn before and after exposure for hemoglobin concentration (Hb). Mean (SD) spleen volume was 279 (115) mL before exposure, 219 (75) mL (21% reduction; $P = 0.005$) at 3 min of exposure, and 201 (93) mL after 10 min exposure to hypoxia (28% reduction; $P < 0.001$). Hb was 138.8 (7.6) $g{\cdot}L^{-1}$ before and 142.9 (8.1) $g{\cdot}L^{-1}$ after 10 min of exposure (2.9% increase; $P < 0.001$). SpO_2 was 96.4 (1.7)% before exposure and 74.7 (8.4)% during the last minute of exposure (22.5% reduction; $P < 0.001$). HR increased from 80 (14) to 90 (17) bpm during exposure (12% increase, $P < 0.05$). MAP remained unchanged. After 10 min recovery on oxygen, values had been restored for spleen volume and Hb, while SpO_2 was higher and HR lower compared with before hypoxia exposure. We concluded that acute normobaric hypoxia of only 10 min caused significant spleen volume contraction with Hb increase. This rapid spleen response, evident already after 3 min of exposure, could have a protective effect during sudden exposure to severe hypoxia.

Keywords: extreme environment, ultrasound, acute survival, high altitude, arterial oxygen saturation

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Annalisa Cogo,
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Reviewed by:
Costantino Balestra,
Haute École Bruxelles-Brabant (HE2B), Belgium
Danilo Cialoni,
Dan Europe Foundation, Italy

***Correspondence:**
Erika Schagatay
Erika.Schagatay@miun.se

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INTRODUCTION

Sudden decreases in oxygen partial pressure can occur in an abrupt loss of cabin pressure in an aircraft (Johnston, 2008; Muehlemann et al., 2013), failure of the oxygen delivery system in high-altitude parachuting (Clemente-Suárez et al., 2017; Ottestad et al., 2017), high-altitude ballooning (Pilmanis and Sears, 2003), or climbing (West, 2003). This is quite different from chronic exposure

where acclimatization plays an important role and also from gradually occurring mild hypoxia. Sudden hypoxemia can also occur in illnesses as acute pulmonary embolism (Fernandes et al., 2019). Another situation is apneic hypoxia, which is associated with several specific responses (Schagatay, 2009) but differs from eupneic hypoxia as it implies the cessation of normal respiration.

The physiological response to acute eupneic hypoxia includes increases in rate and depth of breathing (Cummins et al., 2020), increased heart rate, higher pulmonary vascular resistance, and a reduction in plasma volume (Luks et al., 2021). However, the acute responses to hypoxia are far from fully explained, and several mechanisms contribute to the outcome on the cellular level. The spleen is known for its immunological properties, but it has a variety of functions and can acutely reduce its volume, eliciting increases in hemoglobin concentration (Hb), in response to hypoxia and various stressful stimuli (Stewart and McKenzie, 2002). Hypoxia induced by apnea is associated with elevated Hb, a response not found in splenectomized subjects (Schagatay et al., 2001; Baković et al., 2003). This is an active response (Baković et al., 2003) that has also been observed during high altitude exposure, where the spleen reduces its volume even more with exercise (Sonmez et al., 2007; Engan et al., 2014; Schagatay et al., 2020b). Functional importance is indicated by the observation that spleen volume was negatively associated with symptoms of acute mountain sickness on the ascent in lowlanders (Holmström et al., 2019) and that spleen volume was larger in Sherpa highlanders, especially in those still living on high altitude, compared with lowlanders (Holmström et al., 2020).

There is also evidence of spleen contraction in other conditions associated with cellular hypoxia, like abdominal blunt trauma (Cruz-Romero et al., 2016), severe acute respiratory syndrome (Ding et al., 2003), and drowning (Haffner et al., 1994). Even when there is relative cellular hypoxia due to increased oxygen consumption, the spleen reduces its volume during heavy exercise (Laub et al., 1993; Shephard, 2016; Schagatay et al., 2020b) and during mild exercise like walking in individuals with pulmonary disease (Schagatay et al., 2015). Thus, the spleen seems to have a general role as a potentially protective mechanism against hypoxia, but it has never been studied if and how soon the spleen response occurs during sudden severe eupneic hypoxia.

When the spleen contracts, it releases erythrocytes into the systemic circulation increasing the Hb and oxygen content in the blood (Espersen et al., 2002). The spleen contraction is also evident during moderate normobaric hypoxia as short as 20 min (Richardson et al., 2008; Lodin-Sundström and Schagatay, 2010) where exposure to 12.8% and 14.2% of oxygen evoked spleen contractions of 18% and 16%, respectively. Hypoxia thus seems to be the main trigger of spleen contraction (Richardson et al., 2008) but it was found that eupneic hypoxia for 20 min did not evoke the same magnitude of spleen contraction as did apnea-induced hypoxic hypoxia of the same severity (Lodin-Sundström and Schagatay, 2010), leading to the suggestion that either the associated hypercapnia, the rate of desaturation, or the apnea input itself was involved. It was later shown that the spleen also responded to hypercapnia (Richardson et al., 2012), while the two other inputs have not been further studied.

In activities done at extreme altitudes over 5.500 m, failure of oxygen supply can suddenly expose the body to severe hypoxemia, with a rapid drop in SpO_2. This hypoxia exposure during high-altitude sky diving, aircraft cabin pressure drop, and balloon flights resembles that seen in some maladies and can be shorter and more severe than those studied previously. It is unclear if the spleen response is occurring rapidly enough to make a difference in these situations, and if it evokes an elevation in Hb.

We, therefore, aimed to evaluate the speed and magnitude of spleen contraction and its possible effects on Hb during short sudden exposure to extreme hypoxia and its subsequent recovery on oxygen. We hypothesized that the spleen will contract and elevate Hb under acute severe hypoxia as short as 10 min. We further hypothesized that the response will develop within minutes and resolve within a few minutes on oxygen.

MATERIALS AND METHODS

Participants

Volunteers were 15 adults, eight females and seven males, with a mean (SD) age of 25 (3) years, height 177 cm (males 185, females 168 cm), and weight 76 kg (males 87, females 66 kg). The participants were all healthy non-smokers involved in physical training 6.6 (3.5) h per week. None of the participants had been at an altitude above 2500 m in the past 6 months. The participants received detailed information about the test protocol and signed an informed consent form. The protocol had been approved by the Regional Human Ethics Board of Umeå University, Sweden, and complied with the Helsinki declaration.

Protocol

The participants arrived at the lab after a period of 12 h without any alcohol consumption and 2 h without eating or drinking caffeine-containing beverages before the testing started. Height and weight were measured, and vital capacity was measured in the standing subject in triplicate with the largest volume used (Spirolite 201 spirometer, Vise Medical Co. Ltd, Chiba, Japan). The participant was seated and had a venous catheter (BD Venflon™ Pro, Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden) inserted in an antecubital vein of the left arm after which all other measurement equipment was attached. The protocol, which started after at least 20 min of sitting rest, consisted of three phases: (1) 5 min of normobaric normoxic respiration (PRE), (2) 10 min of normobaric hypoxic respiration of gas containing 10.0% oxygen in nitrogen (Hypoxico, Hypoxico Inc., New York, NY, USA) equivalent to 5,793 m altitude (EXP), and (3) 10 min of normobaric hyperoxic respiration (POST) at 100% oxygen, mimicking rescue oxygen breathing. The different gas mixtures were administered via face mask.

Measurements

Minute-to-minute measurements of spleen size were obtained via ultrasonic imaging (Mindray DP-6600, Shenzhen Mindray Bio-Medical Electronics CO., Ltd., Shenzhen, China) where three-axial spleen diameters were measured. Blood samples (3 mL) were drawn during the last minute of each phase and analyzed

directly for Hb in triplicate (ABx Diagnostics Micros 60 CT, Montpellier, France). In two participants, capillary samples were taken in triplicate, as catheter insertion was not possible, and Hb was analyzed with HemoCue (HemoCue [Hb] 201+, HemoCue AB, Ängelholm, Sweden).

Heart rate (HR) and peripheral oxygen saturation (SpO_2) were measured continuously across all phases with a pulse oximeter with a finger probe (Biox 3700e, Ohmeda, Madison, USA), and mean arterial blood pressure (MAP) was measured with a photoplethysmometer via finger cuff placed on the right hand (Finapress 2300, Ohmeda, Madison, USA). The end-tidal oxygen concentration (EtO_2) and the end-tidal carbon dioxide concentration ($EtCO_2$) in the face mask were measured via a gas analyzer (NormocapOxy, Datex Ohmeda, Helsinki, Finland). To avoid hypoxic syncope in the participants, the minimum SpO_2 accepted was set at 65%; if the value was reached, phase 2 was terminated, and the participant was moved to phase 3. Continuous data were stored in a computer, together with an event-marker, via a BioPac MH100A CE multichannel data acquisition system (Biopac Systems Inc., Goleta, CA, USA) and were analyzed further using AcqKnowledge Software (Biopac Systems Inc, USA). Breath-by-breath data were analyzed for respiratory frequency and inhaled and exhaled oxygen fraction.

Analysis

Spleen volume was calculated with the Pilström equation: $L \times \pi \left(\frac{(W \times T - T^2)}{3}\right)$ using the spleen maximal length (L), width (W), and thickness (T) obtained from the ultrasound measurements (Schagatay et al., 2005). This formula describes the differences between two ellipsoids divided by two, based on a generalized spleen shape. The spleen volume of the individual in the resting normobaric state before exposure was calculated based on the averaged measures during the last 3 min before the start of hypoxia and used as baseline spleen volume. End of exposure spleen volume was the average of the last 3 min of EXP. Recovery spleen volume was the average of the last 3 min of POST.

Statistical Analysis

Participants served as their own controls. The normal distribution of all data was tested using the Shapiro-Wilk test. Paired Student's *t*-test was used to compare PRE and EXP gas values. Assessments of interaction effects between the independent variables (treatment [PRE, EXP; POST]) and sex on the dependent variables (spleen volume, Hb, SpO_2, and HR) were conducted by a two-way mixed ANOVA with Bonferroni corrections for repeated measures. Assessments of associations between dependent variables were conducted with bivariate correlation tests using Pearson product-moment correlation coefficient (*r*). Meaningfulness of effects was estimated by the standardized mean difference (Cohen's *d*, effect size [ES]) computed as the mean difference divided by the pooled SD. ES was presented along with 95% confidence intervals [CI]. Statistically significant difference was accepted at $P < 0.05$. Interpretation of ES was based on three categories: 0.0–0.5 was considered a small effect, 0.6–1.1 was considered a medium effect, and ≥1.2 was considered a large effect.

TABLE 1 | Baseline characteristics divided by sex.

	Male	Female	*P*
Characteristics			
Vital capacity (L)	6.1 (0.5)	4.1 (0.6)	<0.001
Hb ($gr.L^{-1}$)*	142.8 (8.7)	137.0 (6.7)	0.394
Spleen volume (mL)*	353 (104)	204 (71)	0.018

Units: $gr.L^{-1}$ (grams per liter), mL (milliliters).
*$n = 14$.

RESULTS

All participants completed the study protocol. In one participant, due to an unusual shape of the spleen, measurements were not possible, and in another participant, Hb samples could not be obtained. These two participants were withdrawn from the respective analysis and entirely from the correlation analysis. Results are presented as mean ± standard deviation (SD) with *n* indicated for the respective variable.

Males had a larger vital capacity and a larger pre-exposure spleen volume than females but Hb baseline levels were similar between sexes (**Table 1**). The two-way mixed ANOVA revealed a significant treatment effect on all dependent variables (spleen volume, Hb, SpO_2, and HR). There was no two-way interaction effect between males and females on the dependent variables during the experimental intervention, showing that these variables change equally between sexes in response to the hypoxia. Results are therefore presented as mean (SD) for the whole group.

End-Tidal Gas values

The EtO_2 was 98.6 (17.2) mmHg at PRE, and it decreased to 46.5 (6.3) mmHg during EXP ($P < 0.001$; ES = 4.00 [3.26–4.75]). The $EtCO_2$ was 32.7 (4.3) mmHg at PRE, and it decreased to 28.3 (4.7) mmHg during EXP ($P < 0.001$; ES = 0.98 [0.23–1.72]).

Oxygen Saturation

The SpO_2 was 96.4 (1.7)% during PRE, and it decreased to 74.7 (8.4)% by the end of EXP ($P < 0.001$; ES = 3.94 [3.20–4.69]; **Figure 1**). During the last 3 min of POST, SpO_2 increased to 98.8 (1.1)% ($P < 0.001$; ES = 4.56 [3.81–5.31]), which was also higher compared with the PRE value ($P < 0.001$; ES = 1.90 [1.15–2.65]; **Figure 1**).

Cardiovascular Parameters

The HR was 80 (14) beats·min^{-1} during PRE, and it increased to 90 (17) beats·min^{-1} in EXP ($P = 0.046$; ES = 0.64 [−0.11–1.39]; **Figure 1**). The HR decreased to 72 (9) beats·min^{-1} during POST ($P < 0.001$; ES = 1.32 [0.58–2.07]), which was also lower compared with PRE ($P = 0.002$; ES = 0.68 [−0.07–1.43]; **Figure 1**). The MAP was 111 (21) mmHg during PRE. There was no change in MAP during EXP at 113 (33) or POST at 111 (19) mmHg (NS).

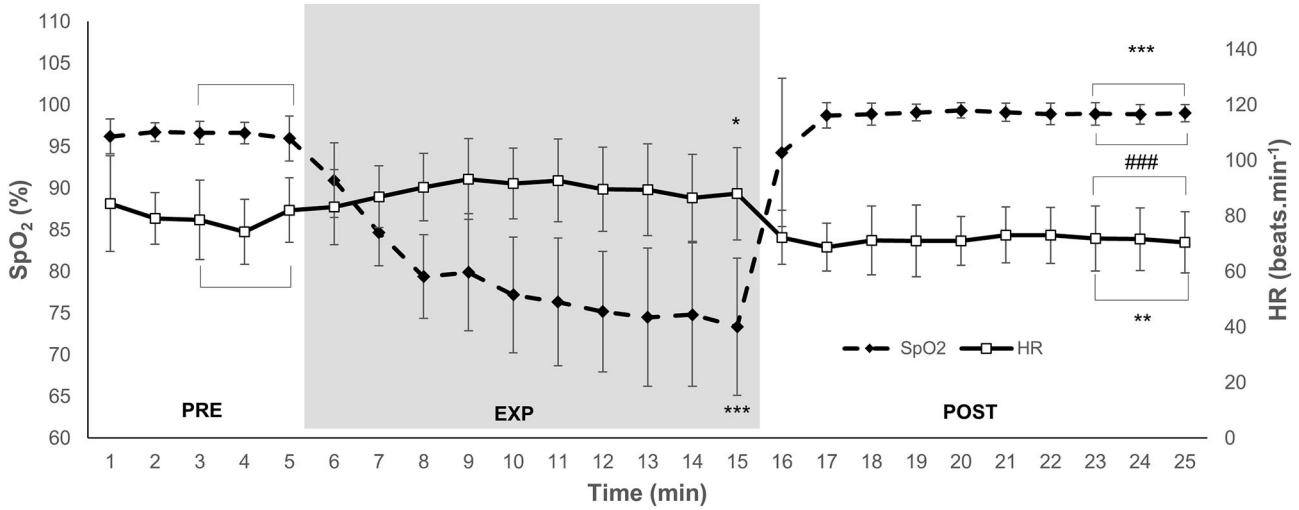

FIGURE 1 | Mean and SD arterial oxygen saturation (SpO_2) and heart rate (HR) during 5 min normoxic breathing (PRE), 10 min hypoxic breathing of 10% O_2 (EXP), and 10 min hyperoxic breathing of 100% O_2 (POST) for $n = 15$. Gray zone indicates EXP. Horizontal brackets indicate an averaged value for that period. Significant difference from (PRE) is indicated by * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$. Significant difference from EXP is indicated by ### for $P < 0.001$.

Spleen Volume

Spleen volume for 14 participants during PRE was 279 (115) mL. After 3 min of EXP, the spleen contracted to 219 (75) mL (by 21%; $P = 0.005$; ES = 0.62 [−0.16–1.39]), and during the last 3 min of EXP, the volume decreased to 201 (93) mL (by 28%; $P < 0.001$; ES = 0.75 [−0.03–1.52]; **Figure 2**). At the end of POST, the spleen volume increased from EXP to 279 (120) mL (with 37%; $P = 0.002$; ES = 0.73 [−0.05–1.50]), which was the same as the PRE value ($P = 1.000$; ES = 0.00 [−0.78–0.78]; **Figure 2**). The individual spleen volume reduction from PRE to EXP ranged between 0 and 134 mL.

Hemoglobin Concentration

The Hb for 14 participants at PRE was 138.8 (7.6) $g{\cdot}L^{-1}$, and after EXP, it was 142.9 (8.1) $g{\cdot}L^{-1}$, which was 4.1 $g{\cdot}L^{-1}$ higher than PRE ($P < 0.001$; ES = 0.52 [−0.26–1.30]; **Figure 3**). Between EXP and 10 min POST, Hb had decreased with 2.7 $g{\cdot}L^{-1}$ to 140.2 (8.0) $g{\cdot}L^{-1}$ ($P = 0.007$; ES = 0.34 [−0.44–1.11]; **Figure 3**). The 10 min POST Hb value was similar to the pre-value ($P = 0.734$; ES = 0.18 [−0.60–0.96]; **Figure 3**). The individual Hb increase from PRE to EXP ranged between 0.2 $g{\cdot}L^{-1}$ and 12.3 $g{\cdot}L^{-1}$.

Correlation Analysis

There was a positive correlation between baseline spleen volume and spleen volume contraction during EXP ($r = 0.692$, $P = 0.006$), but no correlation between initial spleen volume and relative volume change (**Table 2**). There was no correlation between SpO_2 nadir and spleen volume contraction nor SpO_2 nadir and relative change in spleen volume (**Table 2**).

We also found a positive correlation between maximal heart rate at EXP with Hb Δ ($r = 0.762$, $P = 0.002$) and also between SpO_2 Δ and Hb Δ ($r = 0.687$, $P = 0.007$; **Table 2**). No correlation was found between spleen volume contraction and Hb increase (**Table 2**).

DISCUSSION

This study shows that during a short sudden exposure of 10 min to severe hypoxia, the spleen contracts and Hb increases, implying that even during short-term exposure the spleen plays a role in the physiological response to acute hypoxia. Indeed, just after 3 min of hypoxia, there was a spleen volume reduction of 21%, with a maximal response at 28% reached after 7 min, and the response stayed stable during the last 3 min of exposure. Thus, there was a biphasic spleen volume reduction with a faster reduction in the beginning followed by a more subtle reduction associated with the gradual reduction in SpO_2. Such a biphasic spleen response has been reported previously in a study on long apneas (Lodin-Sundström et al., 2009), but it has never been observed previously after eupneic acute hypoxia, and shows that a situation of sudden exposure may induce an early stress-induced spleen contraction, followed by the development of a more powerful response when hypoxia develops. This is contrary to the interpretation done by Palada et al. (2007), which concluded that spleen contraction is induced during just the initial seconds of apnea but without the influence of hypoxia. We instead suggest that both the general sympathetic stimulus and hypoxia contribute to the response associated with hypoxic exposure, at least during eupneic exposure. As the subjects were sitting down and not performing any type of physical activity, hypoxia can be considered the most likely cause of these further adjustments. The spleen contraction had a close relation to the decrease in SpO_2 and the increased heart rate. This suggests that the spleen contraction is an attempt to maintain oxygenation, via elevation of Hb, to sustain body functioning in conditions of sudden hypoxia. Hb elevation, albeit small in the whole group, was highly individual and varied between no effect to 9% increase, and in strong responders, it could be large enough to significantly facilitate oxygen delivery. Concerning the individual variation,

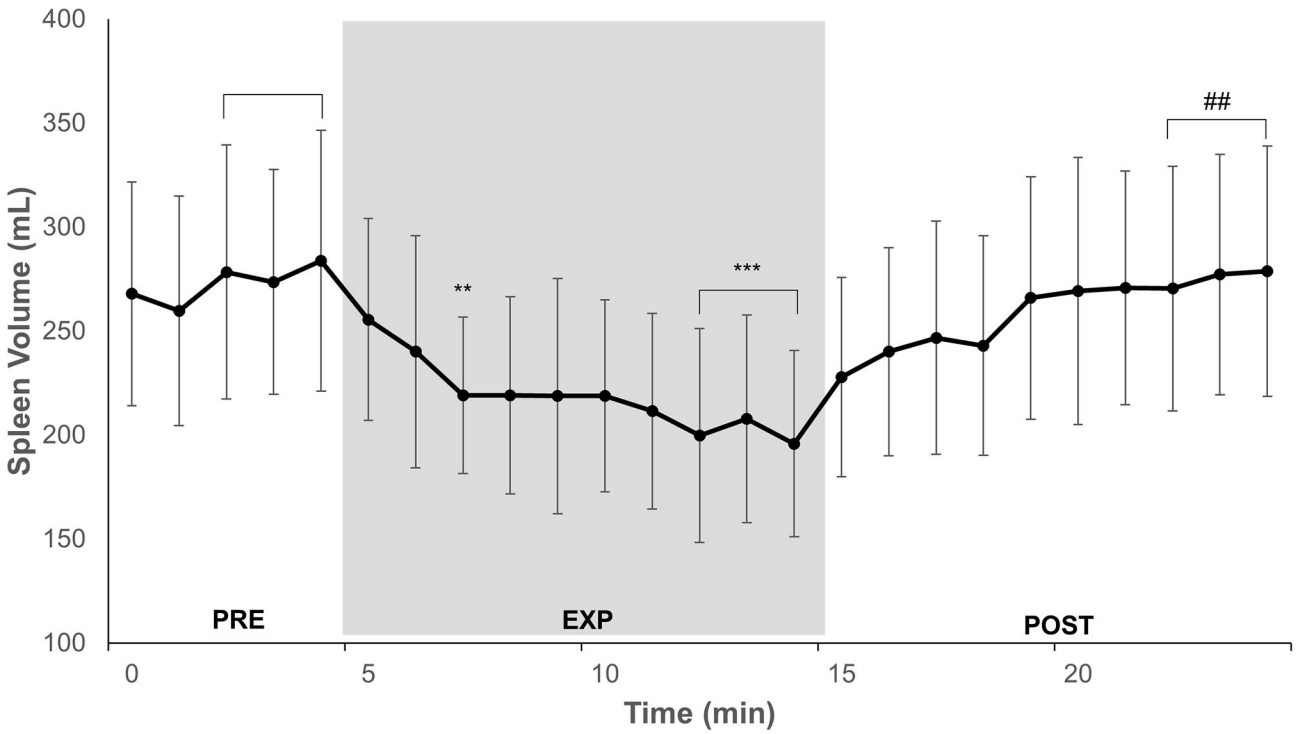

FIGURE 2 | Minute-to-minute mean and SD of spleen volume during 5 min normoxic breathing (PRE), 10 min hypoxic breathing of 10% O_2 (EXP), and 10 min hyperoxic breathing of 100% O_2 (POST) for $n = 14$. Gray zone indicates EXP. Horizontal brackets indicate an averaged value for that period. Significant difference from PRE is indicated by ** for $P < 0.01$ and *** for $P < 0.001$. Significant difference from EXP is indicated by ## for $P < 0.01$.

it is important to notice that one participant did not have any change in spleen size or Hb.

The development of the spleen response was much faster in this situation with simulated 5,793 m exposure than with the exposure to 3,110 m as in the study by Lodin-Sundström and Schagatay (2010). The rapid response occurs within the "time of useful consciousness" (Yoneda et al., 2000) identified in pilots during rapid cabin pressure fall, or failing oxygen systems during flight, which suggests that the response could be useful to prolong conscious time and enable the victim to take action in various hypoxic situations.

The spleen contraction was reversed and Hb restored within 7 min of breathing 100% oxygen, showing that it is a transient response that is fully reversed within minutes when the hypoxic stimulus ceases. This rapid recovery is in line with previous studies of spleen recovery within 10 min after longer normobaric hypoxic exposure (Richardson et al., 2008; Lodin-Sundström and Schagatay, 2010) and within 8–9 min after serial apneas (Schagatay et al., 2005). The underlying mechanism behind the normalizing of Hb after a hypoxic challenge is not fully understood. We speculate that passive enlargement of the spleen with elongation of the contractile elements of the capsule occurs when the spleen red pulp is filled with blood and that a filtering effect leads to a higher Hb in the spleen than in the circulating blood.

The 28% spleen volume reduction in this study was greater in magnitude than the 16% (Lodin-Sundström and Schagatay, 2010) and 18% (Richardson et al., 2008) found in previous studies using milder eupneic hypoxia, suggesting that the severity of hypoxia during sudden exposure is important in determining the magnitude, and thus, it seems like the response is graded.

We used 10.0% oxygen in the inhaled gas, leading to 74.7% SpO_2, which was lower than the 87.2% SpO_2 seen at 14.2% oxygen (Lodin-Sundström and Schagatay, 2010), but higher than the 64.4% at 12.8% oxygen (Richardson et al., 2008), although the last study result can be explained by extreme desaturation in one participant. This might suggest that the splenic contraction is affected not only by the level and duration of exposure but also by the sudden drop in oxygen concentration, in line with the previous suggestion that the rate of desaturation during normobaric hypoxia can be a factor modifying spleen contraction (Lodin-Sundström and Schagatay, 2010). Stewart et al. (2003) reported that the splenic contraction was not affected by the duration of submaximal exercise but lacked information about SpO_2 during the different durations of exercise. A SpO_2 nadir that does not correlate with the decrease in spleen volume suggests that also other factors besides hypoxia may stimulate the contraction. Individual stress response and catecholamine levels play a role in spleen contraction (Stewart et al., 2003; Purdy et al., 2019), and according to the great HR variation during hypoxia in our participants, we can speculate that different individual levels of stress could have affected the spleen response. However, it is in line with previous studies that the spleen response magnitude on the same stimulus can be highly individual (Espersen et al., 2002;

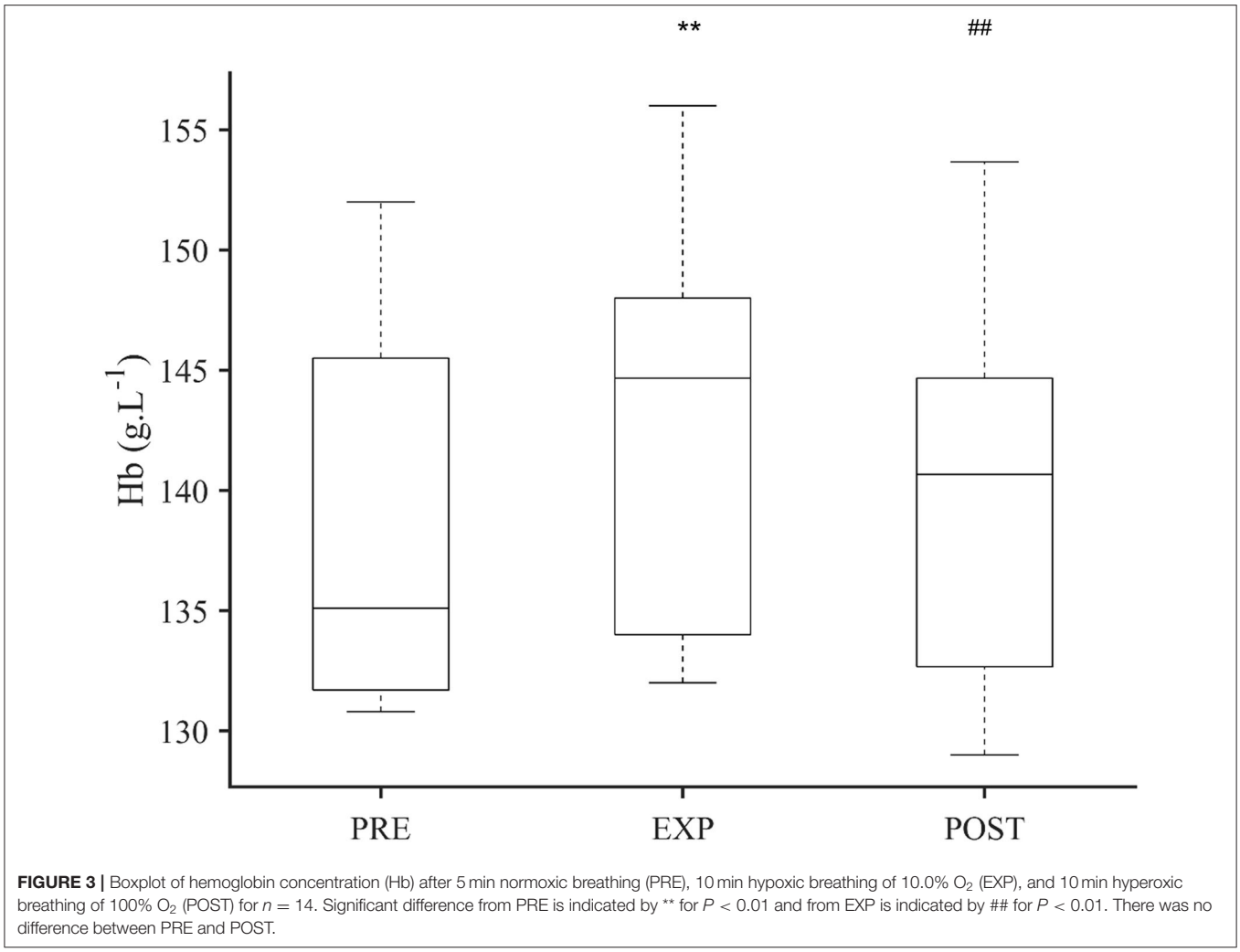

FIGURE 3 | Boxplot of hemoglobin concentration (Hb) after 5 min normoxic breathing (PRE), 10 min hypoxic breathing of 10.0% O_2 (EXP), and 10 min hyperoxic breathing of 100% O_2 (POST) for $n = 14$. Significant difference from PRE is indicated by ** for $P < 0.01$ and from EXP is indicated by ## for $P < 0.01$. There was no difference between PRE and POST.

TABLE 2 | Correlation analysis.

Variable 1	Variable 2	*r*	*P*
HR peak	Hb Δ	0.762	0.002
Spleen	Spleen Δ	0.692	0.006
SpO_2 Δ	Hb Δ	0.687	0.007
SpO_2 nadir	Spleen %	0.445	0.111
Spleen	Spleen %	−0.262	0.366
SpO_2 nadir	Spleen Δ	−0.204	0.483
Spleen Δ	Hb Δ	0.104	0.736

Correlation analysis results for n = 13 participants. Initial spleen volume (Spleen). Spleen reduction from PRE to EXP (Spleen Δ). Spleen relative volume change expressed as a percentage from PRE to EXP (Spleen %). Hb increase from PRE to EXP (Hb Δ). SpO_2 drop from PRE to EXP (SpO_2 Δ). Pearson's correlation factor = r.

Prommer et al., 2007; Engan et al., 2014). The positive correlation that we found between peak HR and Hb increase is interesting, as sympathetic activation could be the common link.

Larger spleens resulted in larger absolute volume contractions in accord with earlier observations (Schagatay et al., 2015; Holmström et al., 2020), while there was no correlation between initial spleen volume and percentage of contraction, showing that individual spleen size is most important for the effect. Thus, individuals with large spleens likely have a greater potential to increase circulating Hb.

The Hb elevation at 4.1 $g{\cdot}L^{-1}$ was within values observed previously: 2.8, 3.5, and 5.4 $g{\cdot}L^{-1}$ during different eupneic hypoxic situations (Richardson et al., 2005, 2008; Schagatay et al., 2020a). While the average Hb response was 4.1 $g{\cdot}L^{-1}$ in the whole group, there was also a great individual variation in this response, with up to 12.3 $g{\cdot}L^{-1}$ Hb increase, and it would be valuable to evaluate this response in a larger sample to reveal differences in strong responders. The positive association between Hb increase and the reduction of SpO_2 at the end of EXP could indicate a greater response in the most hypoxemic individuals; it agrees with the response-initiation by hypoxia and also seems functional as it elevates the arterial content of oxygen and maintains adequate oxygen delivery (Moraga et al., 2018). The Hb increase was, however, not associated with spleen volume or with the magnitude of spleen volume reduction, as found in some previous studies (Espersen et al., 2002; Richardson et al.,

2005; Prommer et al., 2007; Engan et al., 2014). In Richardson et al. (2008), the spleen was estimated to contribute to 60% of the observed overall increase in hematocrit during hypoxic exposure, and thus, the increase in Hb was not only explained by spleen contraction, which may be the case also in this study. Perhaps, as in other animal species, humans can use another blood reservoir organ, e.g., liver and kidneys, when the arterial oxygen content is reduced (Carneiro and Donald, 1977; Xia et al., 2016). In any case, spleen contraction seems to be a major contributor to elevated Hb.

This study adds more information about the acute response of the spleen to hypoxia, which could apply to both exposure to extreme environments and some diseases and warrants further research to explain the underlying mechanisms and functional effects.

Limitations

Measurement of Hb was done only after maximal exposure time, and it would have been informative to collect data also after 3 min when spleen contraction was already evident. The sample is small with great inter-individual variation, as often in this type of experimental studies, which makes correlation analyses unreliable. We did not study changes induced just by the stress of the protocol on the spleen volume, which would be useful for the determination of the details of the underlying mechanisms. Additionally, we did not measure plasma volume that could affect the values of Hb under hypoxia (Beidleman et al., 2017; Young et al., 2019; Schlittler et al., 2021), although we believe this short exposure could not induce significant changes responsible for the observations. In two participants, the hemoglobin concentration was measured on capillary samples that can yield higher results (Rappaport et al., 2020), although they acted as their own controls, and this should not affect the changes studied.

CONCLUSION

We conclude that sudden eupneic hypoxia as short as 10 min caused significant spleen contraction leading to Hb increase, a response nearly twice as powerful as seen with slowly developing hypoxia. We speculate that this rapid spleen response, evident already after 3 min of exposure, could have a protective effect during sudden exposure to severe hypoxia in different situations. The response magnitude was highly individual, and we speculate that this could reflect different tolerance to sudden hypoxia.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, if confidentiality is guaranteed.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Human Ethics Board of Umeå University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FP contributed to the data analysis, interpretation, and manuscript writing. FS contributed to the conception of the study, data acquisition, preliminary analysis, and critical review of the manuscript. CV contributed to the data acquisition, preliminary analysis, and critical review of the manuscript. ES contributed to the conception of the study, data acquisition, data analysis, and manuscript writing. All authors contributed to the article and approved the submitted version.

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REFERENCES

Baković, D., Valic, Z., Eterović, D., Vuković, I., Obad, A., Marinović-Terzić, I., et al. (2003). Spleen volume and blood flow response to repeated breath-hold apneas. *J. Appl. Physiol.* 95, 1460–1466. doi: 10.1152/japplphysiol.00221.2003

Beidleman, B. A., Staab, J. E., Muza, S. R., and Sawka, M. N. (2017). Quantitative model of hematologic and plasma volume responses after ascent and acclimation to moderate to high altitudes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 312, R265–R272. doi: 10.1152/ajpregu.00225.2016

Carneiro, J. J., and Donald, D. E. (1977). Blood reservoir function of dog spleen, liver, and intestine. *Am. J. Physiol.* 232, H67–H72. doi: 10.1152/ajpheart.1977.232.1.H67

Clemente-Suárez, V. J., Robles-Pérez, J. J., Herrera-Mendoza, K., Herrera-Tapias, B., and Fernández-Lucas, J. (2017). Psychophysiological response and fine motor skills in high-altitude parachute jumps. *High Alt. Med. Biol.* 18, 392–399. doi: 10.1089/ham.2017.0071

Cruz-Romero, C., Agarwal, S., Abujudeh, H. H., Thrall, J., and Hahn, P. F. (2016). Spleen volume on CT and the effect of abdominal trauma. *Emerg. Radiol.* 23, 315–323. doi: 10.1007/s10140-016-1402-5

Cummins, E. P., Strowitzki, M. J., and Taylor, C. T. (2020). Mechanisms and consequences of oxygen and carbon dioxide sensing in mammals. *Physiol. Rev.* 100, 463–488. doi: 10.1152/physrev.00003.2019

Ding, Y., Wang, H., Shen, H., Li, Z., Geng, J., Han, H., et al. (2003). The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. *J. Pathol.* 200, 282–289. doi: 10.1002/path.1440

Engan, H. K., Lodin-Sundström, A., Schagatay, F., and Schagatay, E. (2014). The effect of climbing mount everest on spleen contraction and increase in hemoglobin concentration during breath holding and exercise. *High Alt. Med. Biol.* 15, 52–57. doi: 10.1089/ham.2013.1061

Espersen, K., Frandsen, H., Lorentzen, T., Kanstrup, I. L., and Christensen, N. J. (2002). The human spleen as an erythrocyte reservoir in diving-related interventions. *J. Appl. Physiol.* 92, 2071–2079. doi: 10.1152/japplphysiol.00055.2001

Fernandes, C. J., Luppino Assad, A. P., Alves-Jr, J. L., Jardim, C., and De Souza, R. (2019). Pulmonary embolism and gas exchange. *Respiration* 98, 253–262. doi: 10.1159/000501342

Haffner, H. T., Graw, M., and Erdelkamp, J. (1994). Spleen findings in drowning. *Forensic Sci. Int.* 66, 95–104. doi: 10.1016/0379-0738(94)90333-6

Holmström, P., Mulder, E., Lodin Sundström, A., Limbu, P., and Schagatay, E. (2019). The Magnitude of diving bradycardia during apnea at low-altitude reveals tolerance to high altitude hypoxia. *Front. Physiol.* 10:1075. doi: 10.3389/fphys.2019.01075

Holmström, P., Mulder, E., Starfelt, V., Lodin-Sundström, A., and Schagatay, E. (2020). Spleen size and function in sherpa living high, sherpa living low and nepalese lowlanders. *Front. Physiol.* 11:647. doi: 10.3389/fphys.2020.00647

Johnston, M. J. (2008). Loss of cabin pressure in a military transport: a mass casualty with decompression illnesses. *Aviat. Space Environ. Med.* 79, 429–432. doi: 10.3357/ASEM.2153.2008

Laub, M., Hvid-Jacobsen, K., Hovind, P., Kanstrup, I. L., Christensen, N. J., and Nielsen, S. L. (1993). Spleen emptying and venous hematocrit in humans during exercise. *J. Appl. Physiol.* 74, 1024–1026. doi: 10.1152/jappl.1993.74.3.1024

Lodin-Sundström, A., Richardson, M., and Schagatay, E. (2009). "Biphasic spleen contraction during apnea in divers suggests chemoreceptor input," in *35th EUBS Annual Scientific Meeting* (Aberdeen). Available online at: http://www.eubs.org/wp-content/uploads/2019/03/EUBS2009~AbstractBook.pdf (accessed June 09, 2021).

Lodin-Sundström, A., and Schagatay, E. (2010). Spleen contraction during 20 min normobaric hypoxia and 2 min apnea in humans. *Aviat. Space Environ. Med.* 81, 545–549. doi: 10.3357/ASEM.2682.2010

Luks, A. M., Ainslie, P. N., Lawley, J. S., Roach, R. C., and Simonson, T. S. (2021). "Physiologic responses to hypoxia," in *High Altitude Medicine and Physiology*, eds M. P. Ward, J. S. Milledge and J. B. West (London: Routledge), 276–881.

Moraga, F. A., Osorio, J., Calderón-Jofr,é, R., and Pedreros, A. (2018). Hemoconcentration during maximum exercise in miners with chronic intermittent exposure to hypobaric hypoxia (3800 m). *High Alt. Med. Biol.* 19, 15–20. doi: 10.1089/ham.2017.0011

Muehlemann, T., Holper, L., Wenzel, J., Wittkowski, M., and Wolf, M. (2013). The effect of sudden depressurization on pilots at cruising altitude. *in Advances in Experimental Medicine and Biology* 177–183. doi: 10.1007/978-1-4614-4989-8_25

Ottestad, W., Hansen, T. A., Pradhan, G., Stepanek, J., Høiseth, L. Ø., and Kåsin, J. I. (2017). Acute hypoxia in a simulated high-altitude airdrop scenario due to oxygen system failure. *J. Appl. Physiol.* 123, 1443–1450. doi: 10.1152/japplphysiol.00169.2017

Palada, I., Eterović, D., Obad, A., Bakovic, D., Valic, Z., Ivancev, V., et al. (2007). Spleen and cardiovascular function during short apneas in divers. *J. Appl. Physiol.* 103, 1958–1963. doi: 10.1152/japplphysiol.00182.2007

Pilmanis, A. A., and Sears, W. J. (2003). Physiological hazards of flight at high altitude. *Lancet* 362, s16–s17. doi: 10.1016/S0140-6736(03)15059-3

Prommer, N., Ehrmann, U., Schmidt, W., Steinacker, J. M., Radermacher, P., and Muth, C. M. (2007). Total haemoglobin mass and spleen contraction: a study on competitive apnea divers, non-diving athletes and untrained control subjects. *Eur. J. Appl. Physiol.* 101, 753–759. doi: 10.1007/s00421-007-0556-y

Purdy, G. M., James, M. A., Rees, J. L., Ondrus, P., Keess, J. L., Day, T. A., et al. (2019). Spleen reactivity during incremental ascent to altitude. *J. Appl. Physiol.* 126, 152–159. doi: 10.1152/japplphysiol.00753.2018

Rappaport, A. I., Karakochuk, C. D., Hess, S. Y., Whitehead, R. D., Namaste, S. M. L., Dary, O., et al. (2020). Variability in haemoglobin concentration by measurement tool and blood source: an analysis from seven countries. *J. Clin. Pathol.* 0,1–7. doi: 10.1136/jclinpath-2020-206717

Richardson, M. X., De Bruijn, R., Holmberg, H. C., Björklund, G., Haughey, H., and Schagatay, E. (2005). Increase of hemoglobin concentration after maximal apneas in divers, skiers, and untrained humans. *Can. J. Appl. Physiol.* 30, 276–281. doi: 10.1139/h05-120

Richardson, M. X., Engan, H. K., Lodin-Sundström, A., and Schagatay, E. (2012). Effect of hypercapnia on spleen-related haemoglobin increase during apnea. *Diving Hyperb. Med.* 42, 4–9.

Richardson, M. X., Lodin, A., Reimers, J., and Schagatay, E. (2008). Short-term effects of normobaric hypoxia on the human spleen. *Eur. J. Appl. Physiol.* 104, 395–399. doi: 10.1007/s00421-007-0623-4

Schagatay, E. (2009). Predicting performance in competitive apnoea diving. Part I: Static apnoea. *Diving Hyperb. Med.* 39, 88–99.

Schagatay, E., Andersson, J. P. A., Hallén, M., and Pålsson, B. (2001). Selected contribution: role of spleen emptying in prolonging apneas in humans. *J. Appl. Physiol.* 90, 1623–1629. doi: 10.1152/jappl.2001.90.4.1623

Schagatay, E., Haughey, H., and Reimers, J. (2005). Speed of spleen volume changes evoked by serial apneas. *Eur. J. Appl. Physiol.* 93, 447–452. doi: 10.1007/s00421-004-1224-0

Schagatay, E., Holmström, P., Mulder, E., Limbu, P., Schagatay, F. S., Engan, H., et al. (2020a). Spleen volume and contraction during apnea in Mt. Everest climbers and everest base camp trekkers. *High Alt. Med. Biol.* 21, 84–91. doi: 10.1089/ham.2019.0028

Schagatay, E., Hubinette, A., Lodin-Sundström, A., Engan, H., and Stenfors, N. (2015). Exercise induced hemoconcentration following spleen contraction in subjects with COPD. *COPD Res. Pract.* 1, 1–7. doi: 10.1186/s40749-015-0015-9

Schagatay, E., Lunde, A., Nilsson, S., Palm, O., and Lodin-Sundström, A. (2020b). Spleen contraction elevates hemoglobin concentration at high altitude during rest and exercise. *Eur. J. Appl. Physiol.* 120, 2693–2704. doi: 10.1007/s00421-020-04471-w

Schlittler, M., Gatterer, H., Turner, R., Regli, I. B., Woyke, S., Strapazzon, G., et al. (2021). Regulation of plasma volume in male lowlanders during 4 days of exposure to hypobaric hypoxia equivalent to 3500 m altitude. *J. Physiol.* 599, 1083–1096. doi: 10.1113/JP280601

Shephard, R. J. (2016). Responses of the human spleen to exercise. *J. Sports Sci.* 34, 929–936. doi: 10.1080/02640414.2015.1078488

Sonmez, G., Ozturk, E., Basekim, C. C., Mutlu, H., Kilic, S., Onem, Y., et al. (2007). Effects of altitude on spleen volume: sonographic assessment. *J. Clin. Ultrasound* 35, 182–185. doi: 10.1002/jcu.20346

Stewart, I. B., and McKenzie, D. C. (2002). The human spleen during physiological stress. *Sport. Med.* 32, 361–369. doi: 10.2165/00007256-200232060-00002

Stewart, I. B., Warburton, D. E. R., Hodges, A. N. H., Lyster, D. M., and McKenzie, D. C. (2003). Cardiovascular and splenic responses to exercise in humans. *J. Appl. Physiol.* 94, 1619–1626. doi: 10.1152/japplphysiol.00040.2002

West, J. B. (2003). Failure on Everest: the oxygen equipment of the spring 1952 Swiss expedition. *High Alt. Med. Biol.* 4, 39–43. doi: 10.1089/152702903321488960

Xia, M., Chao, Y., Jia, J., Li, C., Kong, Q., Zhao, Y., et al. (2016). Changes of hemoglobin expression in response to hypoxia in a Tibetan schizothoracine fish, Schizopygopsis pylzovi. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 186, 1033–1043. doi: 10.1007/s00360-016-1013-1

Yoneda, I., Tomoda, M., Tokumaru, O., Sato, T., and Watanabe, Y. (2000). Time of useful consciousness determination in aircrew members with reference to prior altitude chamber experience and age. *Aviat. Space Environ. Med.* 71, 72–76.

Young, A. J., Karl, J. P., Berryman, C. E., Montain, S. J., Beidleman, B. A., and Pasiakos, S. M. (2019). Variability in human plasma volume responses during high-altitude sojourn. *Physiol. Rep.* 7:e14051. doi: 10.14814/phy2.14051

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BRIEF RESEARCH REPORT
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Physiological and Clinical Impact of Repeated Inhaled Oxygen Variation on Erythropoietin Levels in Patients After Surgery

Maher Khalife[1]*, *Mohammed Ben Aziz*[1], *Costantino Balestra*[2], *Joseph Valsamis*[3] and *Maurice Sosnowski*[1]

[1] Department of Anesthesiology, Institut Jules Bordet, Université Libre de Bruxelles (ULB), Brussels, Belgium, [2] Environmental and Occupational Physiology Laboratory, Haute Ecole Bruxelles-Brabant, Brussels, Belgium, [3] Universitair Verplegingscentrum (UVC) Brugmann Site Victor Horta, Clinical Biology, Brussels, Belgium

The "Normobaric Oxygen Paradox" (NOP) is a physiologic mechanism that induces an increase of endogenous erythropoietin (EPO) production by creating a state of relative hypoxia in subjects previously exposed to hyperoxia, followed by a rapid return to normoxia. Oxygen exposure duration and inspired oxygen fraction required to observe a significant increase in EPO or hemoglobin are not clearly defined. Consequently, we here study the effect of one model of relative hypoxia on EPO, reticulocytes and hemoglobin stimulation in patients after surgery. Patients were prospectively randomized in two groups. The O_2 group ($n = 10$) received 100% oxygen for 1 h per day for eight consecutive days, via a non-rebreathing mask. The control group ($n = 12$) received no oxygen variation. Serum EPO, hemoglobin and reticulocyte count were measured on admission and postoperatively on days seven and nine. Percentage EPO at day nine with respect to the baseline value was significantly elevated within the groups [O_2 group: 323.7 (SD ± 139.0); control group: 365.6 (SD± 162.0)] but not between them. No significant difference was found between the groups in terms of reticulocytes count and hemoglobin. Our NOP model showed no difference on EPO increase between the two groups. However, both groups expressed separately significant EPO elevation.

Keywords: hematopoiesis, normobaric hyperoxia, normobaric oxygen paradox (NOP), transfusion alternatives, relative hypoxia

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***Correspondence:**
Maher Khalife
maher.khalife@bordet.be

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INTRODUCTION

At the turn of the 21st century, clinicians were optimistic that an alternative to blood transfusion would be developed. This pressing need arose mainly from three factors: a high demand on limited blood supplies, the reluctance of patients to consume blood components and the risk associated with blood transfusion (Vossoughi et al., 2018). Though the best "alternative" to blood transfusions is to prevent and avoid them, this is not always possible. The development of "artificial blood" or a "blood substitute" is under investigation (Azuma et al., 2017; Davis et al., 2018), but there is still no optimal substitute for human blood although other alternatives have been developed (Bursi et al., 2009; Ruhl et al., 2009).

The use of the pharmacological stimulating agent, erythropoietin (EPO), has proven its efficacy in the treatment of chronic anemia. EPO induces red blood cell production by activating red bone marrow progenitor cells, which in turn stimulates reticulocytes (Jelkmann and Jelkmann, 2013). However, several side effects have been reported on its pharmacological administration (Bohlius et al., 2009). Moreover, the price of such medication remains high and its availability for patients in certain countries is limited.

A few years ago, a phenomenon known as the "normobaric oxygen paradox" (NOP) was described. This technique, whereby a high concentration of oxygen (O_2) is given to spontaneously breathing subjects at normobaric pressure, increase significantly the production of endogenous erythropoietin (Balestra et al., 2006, 2010). The mechanism explaining this phenomenon is based on a cellular model adjusted to hypoxia and it depends on the availability of the reactive oxygen species (ROS). The principle element is the complex formed by the hypoxia-inducible factors 2 alpha and 1 alpha (HIF-1α and 2α) and the tumor-suppressing Von Hippel Lindau protein (VHLp), which is constantly bound to ubiquitin ligase (Ivan et al., 2001; Jaakkola et al., 2001). A limited availability or absence of ROS leads to the complex dissociation and dimerization of HIF-1α with HIF-1β. This latter complex induces EPO gene expression and EPO hormone production (Masson et al., 2001).

Much of the literature suggests that the NOP effect could lead to the production of endogenous EPO (Balestra et al., 2006; Burk, 2007; Ciccarella et al., 2011; Cimino et al., 2012). Recently, a clinical study and limited to 48 h has supported the evidence of the NOP effect (Donati et al., 2017). Conversely, Keramidas et al. (2011, 2012) suggest that the NOP effect is not always clear cut. However, it is thought that the specific period of hyperoxia and oxygen concentration is key to induce the effect. Consequently, it might be possible that the proposed regimen was not optimal to induce the NOP effect. Unfortunately, the precise regime need is still unknown.

The present study evaluates whether the application of one NOP regime could efficiently increase EPO level on patients undergoing deep inferior epigastric perforator flap surgery (DIEP).

MATERIALS AND METHODS

Inclusion criteria of this prospective single-center, controlled, randomized study are female patients of a minimum 18 years old eligible for DIEP surgery. Exclusion criteria included: severe renal insufficiency (GFR $<$ 60 ml/min and creatinine $>$ 2 mg/dl), bleeding necessitating iterative transfusion per and/or postoperatively, severe respiratory syndrome requiring constant oxygen administration and an intolerance to wearing an oxygen mask. All samples are collected and processed at the Institut Jules Bordet, Belgium, with the exception of EPO serum, which is sent to the clinical laboratory of CHU-Brugmann.

A total of twenty six patients scheduled for plastic surgery were enrolled between April and November 2017. The study was approved by the local ethics committee of Institut Jules Bordet, Belgium (approval number CE2103). The work was in accordance to the declaration of Helsinki. Written informed consent was obtained from all participants.

Patients are randomized to two parallel groups at the end of the surgery. Randomization is performed using a Microsoft Excel program. The knowledge of the treatment randomization is open to all except for the laboratory staff.

In our study, sample size calculations are not performed, however the sample size is equal or greater to previously published studies (Ciccarella et al., 2011; Cimino et al., 2012).

All patients underwent general anesthesia. During the surgery, oxygen administration was maintained between 40 and 50%.

All patients were free from mechanical ventilation at the end of the surgery and were admitted to the intensive care unit (ICU) for 48 h. During this ICU stay, a baseline oxygen was administered to every patient via a cannula to ensure saturation above 98%. Oxygen variation started on the first day postoperatively between eight and 10 o'clock in the morning. The first group (O_2 group) received 100% oxygen for 1 h per day for eight consecutive days post-surgery, via a non-rebreathing mask. The second group (control group) received no oxygen variation during the postoperative period. All patients received a continuous dose (1 or 2 liters/min.) of Oxygen to reach 98% of saturation for 48 h postoperatively during ICU stay. After ICU discharge, no oxygen administration was necessary in either control or O_2 group since 98% of saturation was reached with spontaneous air breathing. However, clinicals signs and surgical complications evolution were constantly evaluated by the staff involved in the study.

The transfusion threshold was set to hemoglobin 9 g/dl along with clinical signs.

Clinical information collected on each patient included age and body mass index.

Clinical data collected included the times of start and end surgery, blood loss and duration of hospital stay. EPO, hemoglobin, hematocrit, reticulocyte count where recorded. Other laboratory data included platelet count, urea, creatinine, ferritin, transferrin saturation, iron, prothrombin time and activated prothrombin time.

To measure EPO baseline values, venous blood samples were collected at eigh am before exposure to peroperative hyperoxia. Follow up lab samples were collected on day two, three, four and nine postoperatively. EPO analysis was performed using an IMMULITE® 2000 (Siemens), with chemiluminescence. The occurrence of any postoperative complications was also recorded. All data were introduced in a Microsoft office program coming from the hospital electronic medical program. The risk of bias was not assessed at the time of the study.

Study Outcomes

The primary end point of the study was to compare the effects of the NOP by evaluating the EPO percentage mean change from

Abbreviations: NOP, Normobaric Oxygen Paradox; EPO, Erythropoietin; DIEP, Deep Inferior Epigastric Perforator; O_2, Oxygen; ROS, reactive oxygen species; HIF-1α, hypoxia-inducible factor 1 alpha; VHLp, Von Hippel Lindau protein; GFR, glomerular filtration rate; ICU, intensive care unit.

TABLE 1A | Patient characteristics for the O_2 and control groups.

Variable	O_2 group ($n = 10$)	Control group ($n = 12$)	*P*
Age (years)	57.70 ± 8.38	49.25 ± 9.23	0.037
BMI[#] (Kgm^{-2})	27.06 ± 5.03	24.87 ± 4.26	NS[‡]
Duration of surgery (min[§])	654.5 ± 52.6	647.4 ± 56.6	NS
Hospital stay (days)	11.60 ± 2.59	10.00 ± 2.17	NS
Blood loss (ml)	1,082 ± 285	1,160 ± 555	NS

Data given as mean ± SD[*].
[*]Standard deviation.
[#]Body mass index.
[‡]Not significant.
[§]Minutes.

TABLE 1B | Patient laboratory data for the O_2 and control groups.

Variable	Day	O2 group ($N = 10$)	Control group ($N = 12$)	*P*
Platelet (10^9L-1)	D0	221 ± 38	267 ± 51	NS[‡]
	D9	346 ± 88	439 ± 149	NS
Urea (mg.dl-1)	D0	34.70 ± 7.21	28.33 ± 9.69	NS
	D9	20.60 ± 5.92	20.25 ± 6.20	NS
Creatinine (mg.dl-1)	D0	0.85 ± 0.11	0.80 ± 0.13	NS
	D9	0.67 ± 0,08	0.61 ± 0.12	NS
Ferritin (g.dl-1)	D0	136.4 ± 79.4	77.4 ± 56.9	NS
	D9	216.4 ± 91.9	194.2 ± 124.3	NS
Transferrin saturation (%)	D0	30 ± 11	33 ± 13	NS
	D9	16 ± 6	14 ± 5	NS
Iron (g.dl-1)	D0	98.9 ± 32.3	117.8 ± 36.1	NS
	D9	48.5 ± 18.7	46.0 ± 23.1	NS
PTT[§] (%)	D0	103.2 ± 12.7	103.1 ± 6.1	NS
	D9	86.0 ± 7.6	83.7 ± 6.6	NS
aPTT[#] (sec)	D0	25.3 ± 3.1	25.0 ± 1.9	NS
	D9	25.8 ± 3.1	25.3 ± 2.5	NS

Data given as mean ± SD[*].
[*]Standard deviation.
[‡]Not significant.
[§]Prothrombin time.
[#]Activated prothrombin time.
D0, baseline; D9, day 9.

baseline to day nine within and between the two groups. The secondary end points included the comparison of the reticulocyte count, hemoglobin level measured at baseline and day nine as well as any complications could occur.

Statistical Analysis

Standard statistical analyses were performed, including mean and standard deviation for each treatment group. A one-sample paired student *t*-test was used to detect the between- and within-subject treatment difference. Kolmogorov Smirnov tests were performed to assess the normality of the data. Student *t*-test was used for data of patients characteristics, laboratory data as well as the EPO absolute results. Taking the initial value as 100%, percentage changes in EPO were calculated, thereby observing relative changes rather than the absolute values. The raw data are available from the Journal office. An ANOVA test for repeated measures was also applied for the percentage changes in EPO at different days, with Geisser-Greenhouse's epsilon correction for standard deviations. Multiple comparisons were performed by means of Tukey's test. Statistical significance was set at $p < 0.05$.

RESULTS

Twenty-six patients were enrolled in the study. Only twenty-two patients were included in the final analysis; one set of data was missing from the O_2 group and three patients (two in the O_2 and one in the control group) required a transfusion during their hospital stay and so were withdrawn from the study. The clinical characteristics of the two groups are shown in **Table 1A**. With the exception of age, we did not notice any significant difference between the groups.

Also we didn't record significant difference comparing the baseline for the platelet count, urea, creatinine, ferritin, transferrin saturation, iron, prothrombin time and activated prothrombin time to their ninth day values (**Table 1B**).

In absolute values, we noticed more EPO production in the control group than in the O_2 group with a peak level at the third day postoperatively (**Table 2**).

A significant increase of EPO production was noted within the groups but not between them (**Table 3**; **Figure 1A**).

At Day nine and with respect to the baseline, we did not notice any significant difference between the groups in terms of reticulocytes count and hemoglobin (**Figures 1B,C**).

The hemoglobin levels at day two postoperatively were, respectively, 9.92 (SD± 1.113) and 9.94 (SD± 0.738) in the O_2 group and in the control group.

Multiple comparison of the means of percentage of EPO levels at day three, four and nine with respect to day two within each group showed a significant difference, respectively, for O_2 group: $324 \pm 153\%$; $p < 0.05$, 393 ± 119; $p < 0.001$, 324 ± 139; $p < 0.05$. Control group: 466.8 ± 250; $p < 0.01$, 448.2 ± 250; $p < 0.05$, 365.6 ± 162; $p < 0.05$. The group effects (R square) were, respectively, 0.496 in O_2 group and 0.362 in the control group.

We did not identify any significant surgical complications between the groups.

DISCUSSION

The production of EPO between individuals and within individuals varies greatly across the day, which is probably due to each person's circadian rhythm (Balestra and Germonpre, 2012). Given this variation, we choose to use an individual's relative results with respect to their own baseline, rather than absolute values. Since red blood cell transfusion could alter EPO production, we excluded the transfused patients from statistical analysis (Schwarz et al., 2005). Our study was designed to investigate the effect of "relative hypoxia" in a specific NOP model. We noted elevated EPO values in both groups which were significantly different within them but not in between.

TABLE 2 | Evolution over time of EPO in O_2 group and control group expressed as mean ± (SD*).

	Day 0	Day 2	Day 3	Day 4	Day 9
EPO (mIU/ml)					
O_2 group ($n = 10$)	10.38(±5.25)	18.39(±13.3)	28.45(±12.38)	36.33(±12.29)	36.53(±27.21)
Control group ($n = 12$)	9.867(±3.60)	21.99(±9.32)	42.19(±24.07)	39.17(±16.15)	32.15(±8.78)

*Standard deviation.

Although EPO responds in hours post oxygen exposure (Balestra et al., 2006; Ciccarella et al., 2011; Revelli et al., 2013; Kiboub et al., 2018), we intended to go forward in our EPO analysis. The rationale behind this measurement at day nine was to correlate it with the reticulocytes profile since in another clinical setting study by our group, we identified reticulocytes increase (Lafere et al., 2013) and we couldn't measure EPO levels at that time point. Furthermore, administration of NAC alone without oxygen (as opposed to Momeni's study)(Momeni et al., 2011) showed an increase of EPO after 8 days (Zembron-Lacny et al., 2009), therefore this time frame was interesting to be investigated. Contrary to most other studies where EPO is measured on healthy volunteers, in our study, subjects were surgical patients. Consequently, their per and postoperative oxygen intake are determined by clinical needs. In fact, during anesthesia, patients were ventilated with at least 40–50% oxygen concentration. Therefore, both groups were exposed to prolonged oxygen till the end of the surgery.

On the other hand, patients wore nasal cannula delivering a constant flow of oxygen during their 48 h stay in the ICU. Oxygen delivery is part of postoperative routine and is adapted to the patient's needs to achieve 98% pulse oximetric saturation. When designing the study, we did not expect that a small variation could influence EPO stimulation. So, our control group received somehow a mild hyperoxia. In a recently young healthy volunteers study (Fratantonio et al., 2021), authors observed a significant increase, up to 4-fold, in nuclear HIF-1α when mild 1 h hyperoxia (30% oxygen) was applied before return to normoxia which in fact represent a 10% change compared to atmospheric oxygen content. Conversely, when 100% oxygen was applied, nuclear HIF-1α increase was of lesser impact. These results could contribute to the understanding of the increased EPO level in our control group and the absence of significant results with respect to the oxygen group.

Normobaric oxygen, if given too often, might not be effective in stimulating EPO synthesis (De Bels et al., 2011, 2012; Balestra and Germonpre, 2012). A high normobaric oxygen concentration seems to be a limiting factor with regard to increasing nuclear HIF-1α production and is considered to rather induce a shift toward an oxidative stress response (Fratantonio et al., 2021). In a randomized clinical trial enrolling cardiac surgery patients, less consistent results are found when delivering a normobaric oxygen fraction of 100% (Ciccarella et al., 2011) than in those studies utilizing 50% normobaric oxygen. However, the study was limited to 48 h mechanically ventilated patients. Also, Keramidas showed that 100% oxygen is not optimal for healthy volunteers (Keramidas et al., 2011). Furthermore, other authors showed that widening the range of normobaric hyperoxia to normobaric hypoxia suppress EPO stimulation (Debevec et al., 2012).

TABLE 3 | Percentage EPO expressed as mean (±SD*) for each group with respect to baseline value (D0†) at different days across the study period (ANOVA test).

Di‡/D0 x100	Day 2	Day 3	Day 4	Day 9
EPO (%)				
O_2 group	192.1 (±112.1)	323.8 (±153.1)	392.9 (±118.8)	323.7 (±139.0)
***P*-value**	**0.0288**	**0.0013**	**<0.0001**	**0.0019**
Control group	260.6 (±168.3)	466.8 (±250.3)	448.2 (±250.1)	365.6 (±162.0)
***P*-value**	**0.007**	**0.0004**	**0.0005**	**0.0004**

*Standard deviation.
‡Different days over the study period.
†Baseline value.
p-values for within-subject change from baseline.

Anemia induced by blood loss could influence EPO synthesis (Alamo et al., 2016). In our study, EPO synthesis was influenced similarly in term of blood loss since no significant difference was identified. Besides, platelet count, prothrombin time and activated prothrombin time variations were similar in both groups. Moreover, no patient reported menstruations during the experimental period.

Another factor that could have influenced EPO production is aging and renal function (Costa et al., 2014; Panjeta et al., 2015). Although the control group was significantly younger than the O_2 group, renal function was quite similar and consequently this would have little chance to influence EPO synthesis between the groups.

One more factor is the inflammatory reaction induced by surgery. It is thought to increase the iron regulatory peptide hepcidin (Verga Falzacappa et al., 2007) making iron less available. This will compromise the action of EPO (Pak et al., 2006). In the present study, the impact of the inflammatory reaction was not investigated and iron deficiency at the end of the total hospital stay was similar in both groups.

For these above mentioned reasons and probably for other hidden ones, we did not observe any differences between the groups with respect to both the EPO, reticulocyte and hemoglobin levels. However, in a recent study (Khalife et al., 2018) we used a similar protocol but applied a NOP regime with a lower oxygen gradient to patients undergoing abdominal surgery. We observed a statistically significant increase in

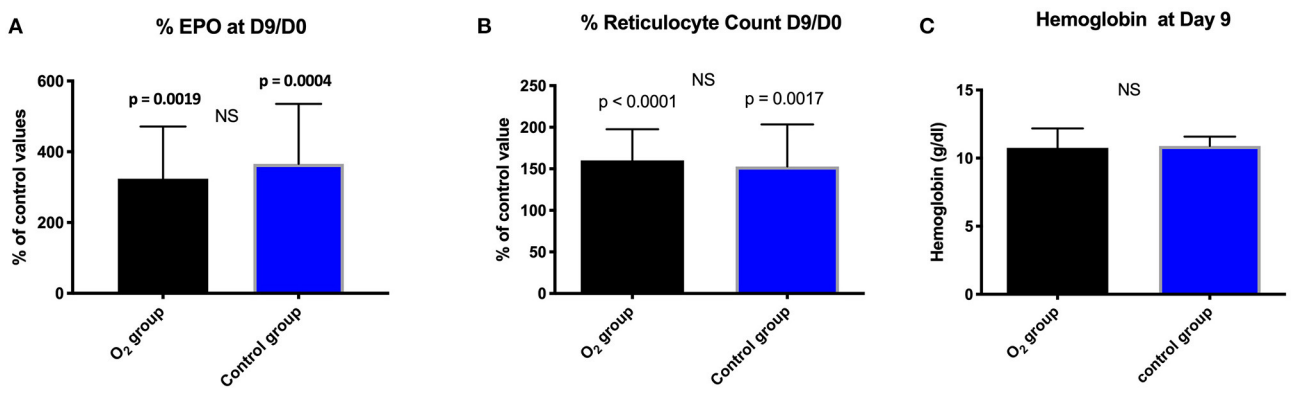

FIGURE 1 | Comparison between and within the groups in percentage: **(A)** EPO; **(B)** Reticulocyte count expressed as mean ± standard deviation at day 9 (D9) with respect to the baseline value (D0); (NS, non-significant). **(C)**: Evolution of hemoglobin (g/dl) expressed as mean ± standard deviation at day 9 in the two groups; (NS, not significant).

reticulocytes in the group exposed to the NOP. Different NOP regime, type of pathology and type of surgery may explain these results. In view of its complexity, we suggest to apply different oxygen concentration in combinations with different oxygen administration intervals in order to explore the phenomenon (Rocco et al., 2014; Fratantonio et al., 2021).

The theory behind the NOP effect mechanism may be understood in two different ways. The first involves glutathione activity, which may be modulated by prolonged hyperoxia thus allowing an EPO rebound, as shown after prolonged hyperoxia during diving (Revelli et al., 2013; Donati et al., 2017). The second explanation depends on the HIF-1alpha increase as proposed by De Bels (De Bels et al., 2011). In this paper, HUVEC cells (human umbilical vein endothelial cells) showed a decrease of HIF-1 alpha expression after 2 h of hyperoxia, reaching 0.59 % of control values, followed, 4 h post hyperoxia, by a reactive increase up to 119.1% and to a 176.6% increase at 6 h post hyperoxia. No absolute hypoxia was applied to the cellular culture lines, so hyperoxia was the only trigger for the increased HIF expression (Cimino et al., 2012). In our study, both mechanisms could be in effect to varying degrees within each group, and this may explain the increase observed in both groups.

In our clinical model, these results lead to debate on several questions: What is the influence of prolonged oxygen administration on EPO stimulation, what is the role of the inflammatory reaction induced by surgery?, is a lower normobaric oxygen concentration or gradient enough for EPO stimulation?, how many repetitions of "relative hypoxia" will produce optimal results?, what time between session will be optimal?

Our study has several limitations. The main one is the small sample size. For clinical purposes, the study is also limited by the constraint normobaric oxygen delivered to both groups. The small size of the sample may of course impact the outcome, nevertheless similar number of subjects were included in other works in clinical setting, even if EPO was not measured (Lafere et al., 2013), we are well aware that the optimal sequence to reach maximal NOP effect is not yet defined, thus a sample to clearly tackle the NOP effect is very difficult to determine particularly after newly published nuclear expression data (Fratantonio et al., 2021). The power calculation according to the percentage outcome in other studies of our group for EPO was already reaching 100% with 12 subjects, if the sample size calculation was on the Hb increase in g/dl (*post-hoc* calculation) we reach a sample of 10 subjects to achieve 80% of power.

The power calculation for the study was difficult to perform since no consensus is reached on the link between EPO level needed to have relevant clinical Hb values (Panjeta et al., 2015). However, neuroprotection and cardioprotection are already described with increased EPO in patients, from exogenous and endogenous origin (Bogoyevitch, 2004). We believe that studies as the one proposed will be useful in the future to fine tune the clinical aspects and determine the future studies design, we have to consider this study as a pilot one. Another limiting factor is our non –anemic population. However, given that both groups showed augmented EPO values during the trial, these limitations could or not be used to argue against any possible influence on the NOP effect. Moreover, very recent data in divers seem to confirm that little PO_2 variations can induce significant EPO variations (Perovic et al., 2020), in fact: the dives performed were 30 meter depth for 30 min once per week for 5 weeks, this is similar to breath 80% of FiO_2 for 30 min. The authors consider that, in their setting, plasma volume variations could also be a triggering factor. In our experiment we cannot invoke such an effect since this parameter was stabilized.

CONCLUSIONS

This randomized study failed to demonstrate a significant difference in EPO production between the two groups. However, it can be inferred from the results that EPO stimulation could probably be influenced by factors other than the NOP and seems to be affected by even a small oxygen fluctuation as well as a prolonged oxygen exposure. Further investigations are needed to determine an optimal threshold, concentration and interval of administration in surgical, non-surgical and anemic patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local Ethics Committee of Institut Jules Bordet, Belgium (approval number CE2103). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MK: protocol designer, collecting results, writing manuscript, data interpretations, and supervisor for the whole project. MB: participation for the running protocol (data discussion and analysis) and oxygen administration. CB: contribution to the design of the protocol, interpretation for the data, and revising the final editing manuscript in all aspects (questions regarding the outcomes correctly investigated). JV: clinical biology analysis. MS: contribution to the design of the protocol, interpretation for the data, and revising the final editing manuscript in all aspects (questions regarding the outcomes and results correctly investigated). All authors contributed to the article and approved the submitted version.

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REFERENCES

Alamo, I. G., Kannan, K. B., Smith, M. A., Efron, P. A., and Mohr, A. M. (2016). Characterization of erythropoietin and hepcidin in the regulation of persistent injury-associated anemia. *J. Trauma Acute Care Surg.* 81, 705–712. doi: 10.1097/TA.0000000000001163

Azuma, H., Fujihara, M., and Sakai, H. (2017). Biocompatibility of HbV: liposome-encapsulated hemoglobin molecules-liposome effects on immune function. *J. Funct. Biomater.* 8:24. doi: 10.3390/jfb8030024

Balestra, C., and Germonpre, P. (2012). Hypoxia, a multifaceted phenomenon: the example of the "normobaric oxygen paradox". *Eur. J. Appl. Physiol.* 112, 4173–4175. doi: 10.1007/s00421-012-2392-y

Balestra, C., Germonpre, P., Lafere, P., and Van der Linden, P. (2010). The Normobaric Oxygen Paradox: a simple way to induce endogenous erythropoetin production and concomitantly raise haemoglobin levels in anaemic patients. *Trans. Alternat. Trans. Med.* 11, 39–42. doi: 10.1111/j.1778-428X.2010.01127.x

Balestra, C., Germonpre, P., Poortmans, J. R., and Marroni, A. (2006). Serum erythropoietin levels in healthy humans after a short period of normobaric and hyperbaric oxygen breathing: the "normobaric oxygen paradox". *J. Appl. Physiol.* 100, 512–518. doi: 10.1152/japplphysiol.00964.2005

Bogoyevitch, M. A. (2004). An update on the cardiac effects of erythropoietin cardioprotection by erythropoietin and the lessons learnt from studies in neuroprotection. *Cardiovasc. Res.* 63, 208–216. doi: 10.1016/j.cardiores.2004.03.017

Bohlius, J., Schmidlin, K., Brillant, C., Schwarzer, G., Trelle, S., Seidenfeld, J., et al. (2009). Erythropoietin or darbepoetin for patients with cancer–meta-analysis based on individual patient data. *Cochrane Database Syst. Rev.* 2009:CD007303. doi: 10.1002/14651858.CD007303.pub2

Burk, R. (2007). Oxygen breathing may be a cheaper and safer alternative to exogenous erythropoietin (EPO). *Med. Hypotheses.* 69, 1200–1204. doi: 10.1016/j.mehy.2007.03.015

Bursi, F., Barbieri, A., Politi, L., Di Girolamo, A., Malagoli, A., Grimaldi, T., et al. (2009). Perioperative red blood cell transfusion and outcome in stable patients after elective major vascular surgery. *Eur. J. Vasc. Endovasc. Surg.* 37, 311–318. doi: 10.1016/j.ejvs.2008.12.002

Ciccarella, Y., Balestra, C., Valsamis, J., and Van der Linden, P. (2011). Increase in endogenous erythropoietin synthesis through the normobaric oxygen paradox in cardiac surgery patients. *Br. J. Anaesth.* 106, 752–753. doi: 10.1093/bja/aer074

Cimino, F., Balestra, C., Germonpre, P., De Bels, D., Tillmans, F., Saija, A., et al. (2012). Pulsed high oxygen induces a hypoxic-like response in human umbilical endothelial cells and in humans. *J. Appl. Physiol.* 113, 1684–1689. doi: 10.1152/japplphysiol.00922.2012

Costa, E., Fernandes, J., Ribeiro, S., Sereno, J., Garrido, P., Rocha-Pereira, P., et al. (2014). Aging is associated with impaired renal function, INF-gamma induced inflammation and with alterations in iron regulatory proteins gene expression. *Aging Dis.* 5, 356–365. doi: 10.14366/AD.2014.0500356

Davis, J. M., El-Haj, N., Shah, N. N., Schwartz, G., Block, M., Wall, J., et al. (2018). Use of the blood substitute HBOC-201 in critically ill patients during sickle crisis: a three-case series. *Transfusion* 58, 132–137. doi: 10.1111/trf.14386

De Bels, D., Corazza, F., and Balestra, C. (2011). Oxygen sensing, homeostasis, and disease. *N. Engl. J. Med.* 365:1845. doi: 10.1056/NEJMc1110602

De Bels, D., Theunissen, S., Devriendt, J., Germonpre, P., Lafere, P., Valsamis, J., et al. (2012). The 'normobaric oxygen paradox': does it increase haemoglobin? *Diving Hyperb. Med.* 42, 67–71. doi: 10.1186/cc9842

Debevec, T., Keramidas, M. E., Norman, B., Gustafsson, T., Eiken, O., and Mekjavic, I. B. (2012). Acute short-term hyperoxia followed by mild hypoxia does not increase EPO production: resolving the "normobaric oxygen paradox". *Eur. J. Appl. Physiol.* 112, 1059–1065. doi: 10.1007/s00421-011-2060-7

Donati, A., Damiani, E., Zuccari, S., Domizi, R., Scorcella, C., Girardis, M., et al. (2017). Effects of short-term hyperoxia on erythropoietin levels and microcirculation in critically ill patients: a prospective observational pilot study. *BMC Anesthesiol.* 17:49. doi: 10.1186/s12871-017-0342-2

Fratantonio, D., Virgili, F., Zucchi, A., Lambrechts, K., Latronico, T., Lafere, P., et al. (2021). Increasing oxygen partial pressures induce a distinct transcriptional response in human PBMC: a pilot study on the "normobaric oxygen paradox". *Int. J. Mol. Sci.* 22:458. doi: 10.3390/ijms22010458

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., et al. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* 292, 464–468. doi: 10.1126/science.1059817

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., et al. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science.* 292, 468–472. doi: 10.1126/science.1059796

Jelkmann, I., and Jelkmann, W. (2013). Impact of erythropoietin on intensive care unit patients. *Transfus. Med. Hemother.* 40, 310–318. doi: 10.1159/000354128

Keramidas, M. E., Kounalakis, S. N., Debevec, T., Norman, B., Gustafsson, T., Eiken, O., et al. (2011). Acute normobaric hyperoxia transiently attenuates plasma erythropoietin concentration in healthy males: evidence against the 'normobaric oxygen paradox' theory. *Acta Physiol.* 202, 91–98. doi: 10.1111/j.1748-1716.2011.02262.x

Keramidas, M. E., Norman, B., Gustafsson, T., Eiken, O., and Mekjavic, I. B. (2012). Long-term intermittent hyperoxic exposures do not enhance erythropoiesis. *Eur. J. Clin. Invest.* 42, 260–265. doi: 10.1111/j.1365-2362.2011.02578.x

Khalife, M., Wiams, K., Ben Aziz, M., Balestra, C., and Sosnowski, M. (2018). Effect of induced relative hypoxia on reticulocyte count in oncologycal abdominal surgery: a single-centre, controlled, randomized pilot study. *Int. J. Sci. Res*. 7, 12–16. doi: 10.36106/ijsr

Kiboub, F. Z., Balestra, C., Loennechen, O., and Eftedal, I. (2018). Hemoglobin and erythropoietin after commercial saturation diving. *Front. Physiol*. 9:1176. doi: 10.3389/fphys.2018.01176

Lafere, P., Schubert, T., De Bels, D., Germonpre, P., and Balestra, C. (2013). Can the normobaric oxygen paradox (NOP) increase reticulocyte count after traumatic hip surgery? *J. Clin. Anesth*. 25, 129–134. doi: 10.1016/j.jclinane.2012.06.021

Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J*. 20, 5197–5206. doi: 10.1093/emboj/20.18.5197

Momeni, M., De Kock, M., Devuyst, O., and Liistro, G. (2011). Effect of N-acetylcysteine and hyperoxia on erythropoietin production. *Eur. J. Appl. Physiol*. 111, 2681–2686. doi: 10.1007/s00421-011-1893-4

Pak, M., Lopez, M. A., Gabayan, V., Ganz, T., and Rivera, S. (2006). Suppression of hepcidin during anemia requires erythropoietic activity. *Blood* 108, 3730–3735. doi: 10.1182/blood-2006-06-028787

Panjeta, M., Tahirovic, I., Karamehic, J., Sofic, E., Ridic, O., and Coric, J. (2015). The relation of erythropoietin towards hemoglobin and hematocrit in varying degrees of renal insufficiency. *Mater. Sociomed*. 27, 144–148. doi: 10.5455/msm.2015.27.144-148

Perovic, A., Zarak, M., Njire Braticevic, M., and Dumic, J. (2020). Effects of recreational scuba diving on erythropoiesis-"normobaric oxygen paradox" or "plasma volume regulation" as a trigger for erythropoietin? *Eur. J. Appl. Physiol*. 120, 1689–1697. doi: 10.1007/s00421-020-04395-5

Revelli, L., Vagnoni, S., D'Amore, A., Di Stasio, E., Lombardi, C. P., Storti, G., et al. (2013). EPO modulation in a 14-days undersea scuba dive. *Int. J. Sports Med*. 34, 856–860. doi: 10.1055/s-0033-1334912

Rocco, M., D'Itri, L., De Bels, D., Corazza, F., and Balestra, C. (2014). The "normobaric oxygen paradox": a new tool for the anesthetist? *Minerva Anestesiol*. 80, 366–372.

Ruhl, H., Bein, G., and Sachs, U. J. (2009). Transfusion-associated graft-versus-host disease. *Transfus. Med. Rev*. 23, 62–71. doi: 10.1016/j.tmrv.2008.09.006

Schwarz, K. B., Dear, P. R., Gill, A. B., Newell, S. J., and Richards, M. (2005). Effects of transfusion in anemia of prematurity. *Pediatr. Hematol. Oncol*. 22, 551–559. doi: 10.1080/08880010500198418

Verga Falzacappa, M. V., Vujic Spasic, M., Kessler, R., Stolte, J., Hentze, M. W., and Muckenthaler, M. U. (2007). STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood* 109, 353–358. doi: 10.1182/blood-2006-07-033969

Vossoughi, S., Perez, G., Whitaker, B. I., Fung, M. K., and Stotler, B. (2018). Analysis of pediatric adverse reactions to transfusions. *Transfusion* 58, 60–69. doi: 10.1111/trf.14359

Zembron-Lacny, A., Slowinska-Lisowska, M., Szygula, Z., Witkowski, K., and Szyszka, K. (2009). The comparison of antioxidant and hematological properties of N-acetylcysteine and alpha-lipoic acid in physically active males. *Physiol. Res*. 58, 855–861. doi: 10.33549/physiolres.931590

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TMT-Based Plasma Proteomics Reveals Dyslipidemia Among Lowlanders During Prolonged Stay at High Altitudes

Pooja[1], *Vandana Sharma*[1], *Ram Niwas Meena*[1], *Koushik Ray*[2], *Usha Panjwani*[2], *Rajeev Varshney*[1] *and Niroj Kumar Sethy*[1]*

[1] *Peptide and Proteomics Division, Defence Institute of Physiology and Allied Sciences, New Delhi, India,* [2] *Neurophysiology Department, Defence Institute of Physiology and Allied Sciences, New Delhi, India*

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Sandro Malacrida, Eurac Research, Italy

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***Correspondence:**
Niroj Kumar Sethy
nksethy@gmail.com;
niroj@dipas.drdo.in

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Acute exposure to high altitude perturbs physiological parameters and induces an array of molecular changes in healthy lowlanders. However, activation of compensatory mechanisms and biological processes facilitates high altitude acclimatization. A large number of lowlanders stay at high altitude regions from weeks to months for work and professional commitments, and thus are vulnerable to altitude-associated disorders. Despite this, there is a scarcity of information for molecular changes associated with long-term stay at high altitudes. In the present study, we evaluated oxygen saturation (SpO_2), heart rate (HR), and systolic and diastolic blood pressure (SBP and DBP) of lowlanders after short- (7 days, HA-D7) and long-term (3 months, HA-D150) stay at high altitudes, and used TMT-based proteomics studies to decipher plasma proteome alterations. We observed improvements in SpO_2 levels after prolonged stay, while HR, SBP, and DBP remained elevated as compared with short-term stay. Plasma proteomics studies revealed higher levels of apolipoproteins APOB, APOCI, APOCIII, APOE, and APOL, and carbonic anhydrases (CA1 and CA2) during hypoxia exposure. Biological network analysis also identified profound alterations in lipoprotein-associated pathways like plasma lipoprotein assembly, VLDL clearance, chylomicron assembly, chylomicron remodeling, plasma lipoprotein clearance, and chylomicron clearance. In corroboration, lipid profiling revealed higher levels of total cholesterol (TC), triglycerides (TGs), low-density lipoprotein (LDL) for HA-D150 whereas high density lipoproteins (HDL) levels were lower as compared with HA-D7 and sea-level indicating dyslipidemia. We also observed higher levels of proinflammatory cytokines IL-6, TNFα, and CRP for HA-D150 along with oxidized LDL (oxLDL), suggesting vascular inflammation and proartherogenic propensity. These results demonstrate that long-term stay at high altitudes exacerbates dyslipidemia and associated disorders.

Keywords: high altitude, hypobaric hypoxia, acclimatization, plasma proteomics, inflammation, dyslipidemia

INTRODUCTION

Human beings experience compromised oxygen delivery at high altitudes ($\geq$ 2,500 m) due to decreased atmospheric pressure and partial pressure of oxygen. This condition of hypobaric hypoxia is the unavoidable, unmodifiable, and uniform environmental stress for everyone at any given altitude. In addition, extreme cold, solar radiation, and aridity are other major stressors for lowlanders at high altitudes, at least for the first few days upon the arrival. Lowlanders elicit an integrated physiological (including respiratory and cardio-pulmonary) and hematological response for acclimatization to high altitude (Houston and Riley, 1947; Bartsch and Gibbs, 2007; Naeije, 2010). It is generally accepted that increased ventilation and hemoconcentration facilitate near normalization of the arterial oxygen content of lowlanders after an approximately 7-day residence at a high altitude (Muza et al., 2010). More recently, several proteins and biochemical pathways have been reported facilitating high altitude acclimatization (Padhy et al., 2016; Tang et al., 2018; Gangwar et al., 2019; Pooja et al., 2020). Failure in such responses may lead to high altitude illnesses, ranging from acute mountain sickness (AMS) to severe and life-threatening forms like high altitude pulmonary edema (HAPE), high altitude cerebral edema (HACE), thromboembolism, and high altitude polycythemia (HAPC) (Reynafarje et al., 1959; Hackett and Roach, 2001; Gallagher and Hackett, 2004; Palmer, 2010; Gupta and Ashraf, 2012). Both animal and human studies have reported that hypobaric hypoxia-induced oxidative stress and inflammation are important factors for the genesis of these high altitude maladies (Hartmann et al., 2000; Sarada et al., 2008; Himadri et al., 2010; Siervo et al., 2014; Boos et al., 2016; Pena et al., 2020a; Pham et al., 2021).

More than 40 million people including thousands of army personnel, government officials, miners, pilgrims, trekkers, and porters visit high altitude regions annually and are at risk of suffering from altitude illness and potentially dying from it (Moore, 2001; Basnyat, 2018). The working conditions and duration of high altitude residence differ among lowlanders depending on their professional requirements. On average trekkers, porters, sportsperson, pilgrims, and tourists spend few days to weeks at high altitudes whereas military personnel, diplomats, miners, and scientists spend months to years at high altitudes. Both acute and chronic exposure to high altitude induces several metabolic alterations and adjustments (Murray, 2016; Murray et al., 2018). More specifically, hypobaric hypoxia exposure alters lipid profiles (Gangwar et al., 2019) inducing hypercholesterolemia and hypertriglyceridemia (Mohanna et al., 2006; Sherpa et al., 2011; Vats et al., 2013) and associated cardiovascular disorders above 3,000 m (Virues-Ortega et al., 2009; Mallet et al., 2021). In addition, extended stay of young and healthy lowlanders at high and extreme altitudes is reportedly associated with a higher risk of spontaneous vascular thrombosis (Anand et al., 2001), massive infarcts, and stroke (Jha et al., 2002). Hence, studying lowlander molecular response above 3,000 m and extended residence period (weeks to months) is a pertinent scientific endeavor that can save both money and manpower.

Blood plasma is a highly accessible sample for monitoring the health status of an individual (Geyer et al., 2016), and high-throughput plasma proteome analysis is a popular omics method to investigate system-level protein alterations that may be rooted from basic science or clinical perspective (Pernemalm and Lehtio, 2014; Ignjatovic et al., 2019). Several research groups including ours have used plasma proteomics for gaining insight into high altitude acclimatization (Ahmad et al., 2011, 2013; Levett et al., 2011, 2015; Julian et al., 2014; Yang et al., 2014; Lu et al., 2018; Wang et al., 2019) and high altitude disorders like AMS (Julian et al., 2014; Lu et al., 2018), HAPE (Ahmad et al., 2011; Yang et al., 2014), polycythemia (Wang et al., 2019), and CMS (Zhang et al., 2021). Despite these studies, no information is available for plasma proteome level alterations of lowlanders during prolonged (months) stay at high altitude. Hence, we sought to investigate global plasma proteome alterations of lowlanders during a 3-month stay at 4,176 m as compared to 7 days stay at high altitude and sea level. In addition, we have also monitored physiological indices, proinflammatory cytokines, and lipid profiles. To the best of our knowledge, this is the first plasma proteomics investigation reporting molecular pathways perturbed during prolonged exposure to high altitude. We have further used biochemical estimation, ELISA studies, and Western blot analysis to validate our proteomics observations. Our present results indicate that acute exposure to high altitude induces dyslipidemia among lowlanders chronic exposure.

MATERIALS AND METHODS

Study Groups

We studied 105 healthy, nonsmoking, male military volunteers (age: 22–37 years, height: 170 $\pm$ 4 cm, weight: 64 $\pm$ 3 kg) at sea level (Pathankot, India, altitude 331 m). All the volunteers are lowlanders-born, living at sea level, and have not been exposed to high altitude for the last 1 year. Subsequently, all the volunteers traveled to Leh (altitude 3,520 m) by road and stayed there for 7 days (high altitude–day seven group, (HA-D7 group; $n = 55$). Then, the volunteers spent 1 day reaching Fukche (4,176 m) and were stationed there for 3 months (high altitude–day 150 group (HA-D150 group; $n = 40$). All the volunteers followed the same diet regimen during the entire study period. The present study was performed according to the Declaration of Helsinki, and the experimental design and procedures for conducting the experiment were approved by the institutional ethics committee (IEC/DIPAS/B2/1). Informed written consent was obtained from all the participants and each volunteer was informed of the possible risk and discomforts involved in the study. During the whole study duration, regular medical examinations were performed to determine health status.

Evaluation of Physiological Parameters

Physiological parameters including oxygen saturation (SpO_2), heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were recorded at sea level and regularly at high altitudes. SpO_2 was measured using a pulse oximeter (Smart Oxy Lite, BPL, India) from the right index finger as the average of three readings. Systolic blood pressure (SBP)

and diastolic blood pressure (DBP) were recorded using a standard sphygmomanometer.

Collection of Blood Plasma

Overnight fasting venous blood samples were drawn in EDTA vacutainer at Pathankot (sea level), Leh (HA-D7), and Fukche (HA-D150 groups). Plasma was separated by centrifugation at 1,500 x *g* for 15 min at 4°C and was stored at −80°C with mammalian protease inhibitor (P8340, Sigma-Aldrich) for further studies.

TMT-Labeling and Plasma Protein Profiling

High abundant plasma proteins from each group (Sea level, HA-D7, and HA-D150) were depleted using High-Select™ Top14 Abundant Protein Depletion Resin (A36370, Thermo Scientific) according to the instructions of the manufacturer. An equal amount (100 μl) of the depleted sample ($n = 8$) was pooled together to comprise 800 μl of total volume for each group. Total protein content was determined with the ToPA Bradford Protein Assay kit (Cat No. K-0014, ITSI Biosciences, USA), and the consistency of protein profiles was checked using SDS-PAGE for each group. Subsequently, a 400 μg depleted protein sample was mixed with 75 μl of 8 M urea buffered in 1 M tetraethylammonium bromide (TEAB). The sample reduction was performed with 5 mM DL-dithiothreitol (DTT) for 30 min at 56°C, followed by alkylation with 11 mM iodoacetamide (IAM) for 15 min at room temperature in darkness. The sample was diluted by adding 200 mM TEAB and digested at a trypsin-to-protein mass ratio of 1:50 for the first digestion overnight and 1:100 for a 4-h digestion. Finally, each group of digested peptides was labeled with the TMT reagents following manufacturer protocols (Thermo Fisher Scientific, Torrance, CA, USA). Sample labeling was as follows: Sea level: 128, HA-D7: 129, and HA-D150: 130.

For multidimensional protein identification technology (MudPIT) analysis, all the labeled samples were combined and then fractionated by strong cation exchange (SCX). The fractions eluted at 50, 75, 150, 250, and 450 mM of ammonium acetate were collected. The samples were desalted using ZipTip, the desalted sample were dried in a speedvac, then resuspended in the appropriate mobile phase. For LC-MS/MS, the chromatography was performed with a Thermo EASY-nLCsystem. Peptides were eluted from the column using a linear acetonitrile gradient from 5 to 32% acetonitrile over 90 min followed by high and low organic washes for another 20 min coupled to Q Exactive™ mass spectrometer (Thermo Scientific) *via* a nanospray source with the spray voltage set to 2.0 kV and the ion transfer capillary set at 250°C. A data-dependent top 15 method was used where a full MS scan from m/z 350–1,600 was followed by MS/MS scans of the 15 most abundant ions. Each ion was subjected to Higher energy C trap dissociation (HCD) for fragmentation, peptide identification, and TMT reporter ion detection. Raw data files of each SCX fraction were searched against the most recent database for humans downloaded from UniProt using the MudPIT option in Proteome Discoverer 2.2 (Thermo Scientific) and the Sequest HT search algorithm. For protein identification results, only peptides identified with high confidence were used. For confidence, the Percolator algorithm was used for peptide spectrum match validation in database searches. The false discovery rate (FDR) threshold calculated in Proteome Discoverer Percolator with high confidence peptides (0.01) were used for protein identification.

Pathway and Network Analysis

The identified proteins were analyzed according to GO terms for biological process, cellular component, and molecular function using the Reactome database (http://www.reactome). Pathway enrichment analysis was assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/ or http://www.kegg.jp/).

Validation of Protein Levels by Immunoblotting

The protein levels in study groups were further validated with immunoblot analysis. Plasma containing 20 μg of protein was separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked in 5% BSA blocking buffer in PBS-0.1% Tween-20 (PBST) overnight at 4°C. Subsequently, the membranes were washed with PBST three times for 5 min each. It was followed by incubation with the Apo-B (PA5-86101, *Invitrogen*), Apo-CIII (701238, Thermo Fischer Scientific), and β-tubulin (MA516308, *Invitrogen*) for 2 h and secondary antibodies for 1.5 h at room temperature, respectively. The blots were washed again with PBST three times for 5 min each and were observed by adding chemiluminescent peroxidase substrate (Cat. No. 34095, ThermoFisher Scientific, USA) on UVP Biospectrum. Densitometry analysis was performed using Image J software.

Lipid Profiling

Plasma lipid parameters including cholesterol, HDL, LDL, and triglycerides were analyzed on a Randox Monaco clinical chemistry analyzer (Randox Laboratories, Crumlin, UK).

Evaluation of Inflammatory Cytokines

The plasma CRP level was evaluated using a human C-reactive protein ELISA Kit (E0829h, EIAab Science, Wuhan, CHINA) as per the instructions of the manufacturer. The levels of IL-6, TNFα, and ox-LDL were evaluated using Human IL-6 PicoKine™ ELISA Kit (EK0410, Boster Biologicals), Human TNFα ELISA Kit (950.090.096, Diaclone, Besancon Cedex, France), and human oxidized low density lipoprotein (OxLDL) ELISA Kit (E-EL-H0124, Elabscience), respectively, as per the recommendations of the manufacturer.

Statistical Analysis

All the values were represented as mean ± SD. Statistical analysis was performed using ANOVA with Newman–Keuls *post-hoc* tests, and a p-value of < 0.05 was considered significant. All analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, California, USA). Pearson correlation analysis was performed for depicting the correlation between physiological and biochemical parameters.

TABLE 1 | Physiological parameters of volunteers at sea level ($n = 105$) who were subsequently exposed to HA for 7 days (HA-D7, $n = 55$) and 3 months (HA-D150, $n = 40$).

	Sea level	HA-D7	HA-D150
SpO_2	98.99 ± 0.001	87.47 ± 2.85***	95.42 ± 0.78∧ ∧ ∧
HR (bpm)	76.60 ± 8.26	77.51 ± 12.09	83.14 ± 12.81∧ ∧ ∧
SBP (mmHg)	118.0 ± 8.97	120.68 ± 10.91	127.44 ± 12.99∧ ∧ ∧
DBP (mmHg)	78.40 ± 3.44	79.69 ± 8.65	84.55 ± 9.16∧ ∧ ∧
Hb (g/dL)	14.07 ± 0.16	15.91 ± 0.62**	15.30 ± 1.05*

*represents $p < 0.05$ as compared to sea level, **represents $p < 0.01$ as compared to sea level, ***represents $p < 0.001$ as compared to sea level, ∧ ∧ ∧ represents $p < 0.001$ as compared to HA-D7.

RESULTS

Evaluation of Physiological Parameters

Physiological parameters including SpO_2, HR, SBP, DBP, and Hb were recorded for all the volunteers at sea level (lowlander) and also at HA (HA-D7 and HA-D150), and are represented in **Table 1**. Ascent to HA resulted in a decrease in SpO_2 level for both HA-D7 (87.47 ± 2.85, $p < 0.001$) and HA-D150 (95.42 ± 0.78) as compared with sea level (98.99 ± 0.001). Comparison between HA-D7 and HA-D150 revealed an 8% increase ($p < 0.001$) of SpO_2 during a long-term stay at HA. The HR increased upon HA stay, irrespective of duration, and the highest HR was observed for HA-D150 (83.14 ± 12.81 bpm, $p < 0.001$) as compared with both sea level (76.60 ± 8.26 bpm) and HA-D7 (77.51 ± 12.09 bpm). Similarly, SBP and DBP also increased upon HA ascent, and HA-D150 possessed the highest SBP (127.44 ± 12.99 mmHg, $p < 0.001$) and DBP (84.55 ± 9.16 mmHg, $p < 0.001$) as compared with both lowlander and HA-D7. HA exposure also resulted in increased hemoglobin for both HA-D7 (15.91 ± 0.62 g/dL, $p < 0.01$) and HA-D150 (15.30 ± 1.05, $p < 0.05$) as compared with lowlanders (14.07 ± 0.16 g/dL).

Plasma Proteomics Study

Plasma proteomics studies were performed to identify global perturbations in plasma proteins and pathways during short- and long-term stay at HA. Plasma was first depleted for abundant proteins followed by TMT labeling and LC-MS/MS-based quantitative proteomics studies. A total of 17,336 peptides were identified in this experiment including low confidence peptides. However, with higher stringency (0.01% FDR), a total of 377 differentially abundant proteins were identified (**Figure 1A**; **Supplementary Table 1**). The frequency distribution of the 377 quantitative proteins, log2-transformed ratios fitted normality distribution (**Figure 1B**). For the HA-D7 group, a total of 75 proteins were found to be downregulated (< 0.8-fold) and 62 proteins were found to be upregulated (>1.2-fold), whereas 56 proteins were downregulated (<0.8-fold) and 79 proteins were upregulated (>1.2-fold) for HA-D150. Comparing both HA-D7 and HA-D150, we observed 32 downregulated proteins (<0.8-fold) and 27 upregulated proteins (>1.2-fold) common between short- and long term stay at high altitude (**Supplementary Tables 2**, **3**).

Identified proteins were uploaded to a curated database "Reactome" (https://reactome.org) to identify underlying pathways altered during high altitude exposure. Interestingly, the top 25 significant pathways identified from common upregulated proteins at high altitude included plasma lipoprotein assembly, VLDL clearance, chylomicron assembly, chylomicron remodeling, plasma lipoprotein clearance, and chylomicron clearance (**Figure 1C**). In corroboration, we observed higher levels of apolipoproteins like APOB (1.32-fold), APOCI (1.36-fold), APOCIII (1.97-fold), APOCIV (1.34-fold), APOCIV + APOCII (1.15-fold), APOE (1.41-fold), and APOL (1.42-fold) for HA-D7. Similarly, we observed higher levels of Apo-AII (1.35-fold), Apo-B (1.34-fold), Apo-CI (1.39-fold), Apo-CIII (1.79-fold), Apo-CIV (1.17-fold), Apo-CIV + Apo-CII (1.20-fold), Apo-E (1.35-fold), and Apo-L (1.36-fold) for HA-D150. Comparing both the groups, we observed higher levels of carbonic anhydrase 1 (CA1, 2.30-fold) and carbonic anhydrase 2 (CA2, 2.36-fold) for HA-D150. We also observed a lower abundance of several cytoskeletal proteins like Profilin-1 (PROF-1), actin, cytoplasmic 2 (ACTG1), Talin-1 (TLN1), tubulin alpha chain, tubulin beta chain, myosin-9 (MYH9), and alpha-actinin-1 (ACTN1) in both the study groups (**Supplementary Tables 1**, **3**).

Validation of Plasma Proteomics Analysis

Validation of protein levels by western blot revealed that APOB levels were higher in HA-D7 (1.33-fold) and HA-D150 (2.36-fold) as compared with sea level. Similar higher levels of Apo-CIII (5.06-fold for HA-D7, $p < 0.001$, and 2.17-fold for HA-D150) were observed as compared to sea level (**Figures 2A,B**).

Altered Lipid Profile During a Prolonged Stay at HA

To support the proteomics-based observations of altered apolipoproteins, lipid profiles of all the study groups were evaluated (**Table 2**). Elevated levels of plasma total cholesterol (225.63 ± 83.38 mg/dL, $p < 0.05$), LDL (135.1 ± 72.53 mg/dL, $p < 0.05$) and triglycerides (147.23 ± 76.74 mg/dL, $p < 0.001$) were observed for HAD-150 as compared with sea-level values. Concomitantly, higher plasma LDL level (135.1 ± 72.53mg/dL, $p < 0.05$) and lower HDL level (41.75 ± 4.98 mg/dL) was observed for HA-D150 as compared with sea-level values (112.17 ± 37.3 mg/dL and 47.19 ± 8.32 mg/dL, respectively). Consequently, the LDL/HDL ratio and total cholesterol/HDL ratio were elevated for HA-D150 (3.24 ± 14.58 and 5.40 ± 16.76, respectively) as compared to both HA-D7 and sea level. Moreover, the atherogenecity index of plasma (AIP) represented as log10 (TG/HDL-C) was also found to be highest in HA-D150 (**Table 2**).

Evaluation of Inflammatory Markers

We evaluated levels of inflammatory markers CRP, IL-6 and TNFα along with ox-LDL in all the study groups. We observed higher CRP levels in altitude exposed groups (1.99-fold for HA-D7, $p < 0.05$ and 1.79-fold for HA-D150) as compared to sea level values (**Figure 3D**). We observed similar higher

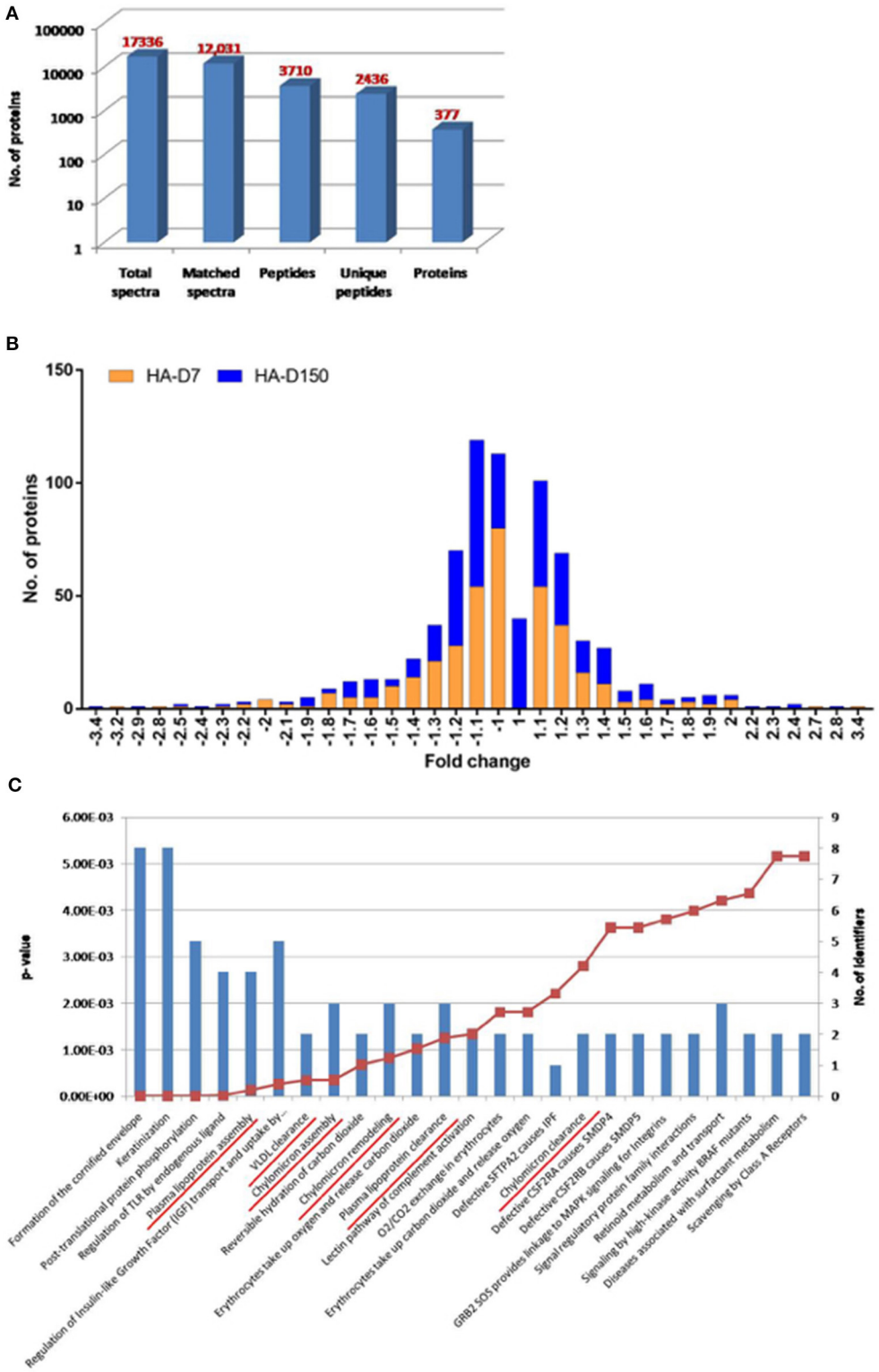

FIGURE 1 | TMT based plasma proteomics analysis. **(A)** Bar graph representing spectra, peptides, and proteins identified from TMT-based LC-MS/MS. **(B)** The quantitative ratio histogram of quantitative proteins identified in the study groups (HA-D7 and HA-D150 with respect to sea level). **(C)** Top 25 significant pathways for the identified common upregulated proteins in HA-D7 and HA-D150 groups representing a number of identified proteins along with their *p*-value. Underlined signify identified pathways involved in lipoprotein metabolism.

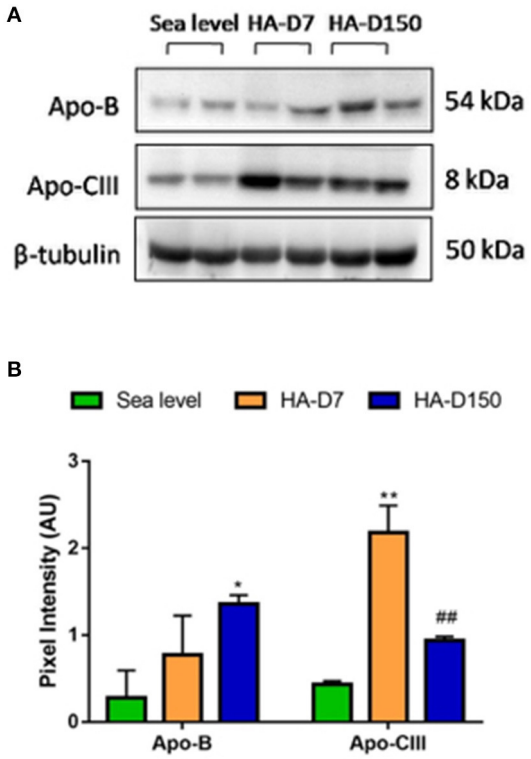

FIGURE 2 | Validation of plasma proteomics data ($n = 6$). **(A)** Western blot analysis of Apo-B and Apo-CIII along with tubulin as a loading control. **(B)** Densitometry analysis of Apo-B and Apo-CIII with respect to tubulin. *represents $p < 0.05$, **represents $p < 0.01$ with respect to sea level and ##represents $p < 0.01$ with respect to HA-D7.

levels of IL-6 and TNFα for HA-D7 (1.97-fold, $p < 0.01$ and 1.11-fold, $p < 0.05$) and HA-D150 (2.11-fold, $p < 0.01$ and 1.14-fold, $p < 0.01$) as compared to sea level values (**Figures 3A,B**). Interestingly, plasma level of ox-LDL was 2.29-fold ($p < 0.001$) and 2.18-fold ($p < 0.001$) higher in HA-D150 (4626 ± 520 pg/ml) as compared to sea level values (2018 ± 256 pg/ml) and HA-D7 (2119 ± 419.8 pg/ml) respectively (**Figure 3C**).

DISCUSSION

The present study reports the physiological and plasma proteome level changes for lowlanders during short- and long-term stay at high altitudes. Comparing the short-term and long-term plasma proteomes with sea level plasma proteome, the present study highlights activation of proatherogenic lipoproteins at high altitude that exacerbates during longer stay durations.

The arterial oxygen saturation level of lowlanders decreased upon acute exposure to high altitude (HA-D7) and increased significantly after long-term stay (HA-D150), though the levels were lower than sea level. This signifies healthy acclimatization to HA (Calbet et al., 2003; Soria et al., 2016) as severe and prolonged oxygen desaturation can lead to AMS (Mandolesi et al., 2014). As expected, the hemoglobin levels also increased significantly at high altitudes and persistently high levels were maintained irrespective of the duration of the stay at HA. Both the systolic and diastolic blood pressure along with heart rate increased upon HA exposure and was more pronounced during a long-term stay at HA. This is majorly due to massive activation of the sympathetic nervous system, contributed by noradrenaline despite enhanced hemoglobin levels and concomitant improved arterial oxygen content (Bartsch and Gibbs, 2007; Hainsworth et al., 2007).

Our TMT-based plasma proteomics studies identified a differential abundance of 377 proteins in HA-D7 and HA-D150 as compared to sea level. Using a cut-off of 0.8-fold for downregulation and 1.2-fold for upregulation, we identified 75 and 56 down-regulated proteins for HA-D7 and HA-D150 groups respectively. Similarly, we identified 62 and 79 upregulated proteins for HA-D7 and HA-D150 groups respectively. Bioinformatics analysis revealed alterations in several lipoprotein-associated pathways like plasma lipoprotein assembly, VLDL clearance, chylomicron assembly, plasma lipoprotein clearance, and chylomicron clearance. We observed higher levels of lipoproteins like APOB, APOCI, APOCIII, APOE, and APOL for both HA-D7 and HA-D150 in accordance with our previous studies (Gangwar et al., 2019). Interestingly, we observed higher levels of Apo-B for both HA-D7 and HA-D150 representing higher levels of low-density lipoprotein (LDL). Findings from several large studies indicate that elevated triglyceride (TG) levels along with increased levels of small dense LDL particles with concomitantly decreased levels of HDL cholesterol are often a component of atherogenic dyslipidemia (Reiner, 2017). We also observed similar high levels of APOCI, the endogenous inhibitor of cholesterol ester transfer protein (CETP) that limits the exchange of lipids has reportedly enhanced the risk of atherosclerosis (Westerterp et al., 2007). Our plasma proteomics and western blot studies identified higher APOCIII levels in high-altitude exposed groups. APOCIII is now recognized as a key regulator in severe hypertriglyceridemia due to its inhibition of lipoprotein lipase (LPL) and hepatic lipase. ApoCIII gain of function mutations are associated with atherosclerosis and coronary heart disease (CHD) and contribute to the development of hypertriglyceridemia, whereas loss of function mutations are associated with lower levels of plasma triglycerides and attenuation of vascular inflammatory processes (Pollin et al., 2008; Tg et al., 2014; Natarajan et al., 2015; Rocha et al., 2017).

Lipid profiling revealed higher levels of cholesterol, triglycerides, LDL, and lower levels of HDL were observed for HA-D150 as compared to HA-D7 and sea level groups (**Table 2**). It is noteworthy that sea-level values for total cholesterol, triglycerides (TGs), and LDL were within the physiological range, increased after high altitude exposure, and levels were further elevated during the prolonged stay. We also observed a moderate correlation of diastolic blood pressure (DBP) with LDL (R-squared = 0.46, $p = 0.04$) for

TABLE 2 | Assessment of lipid profile at sea level, 7 days, and 3 months ($n = 8$).

	Sea level	HA-D7	HA-D150
Triglycerides (mg/dL)	82.29 ± 31.04	105.62 ± 46.39	147.23 ± 76.74***
Total Cholesterol (mg/dL)	186.46 ± 50.08	175.21 ± 63.01	225.63 ± 83.38*∧
HDL (mg/dL)	47.19 ± 8.32	49.35 ± 14.90	41.75 ± 4.98
LDL (mg/dL)	112.17 ± 37.30	76.54 ± 25.25*	135.1 ± 72.53*∧
LDL/HDL ratio	2.38 ± 4.48	1.55 ± 1.69	3.24 ± 14.58* ∧ ∧
Cholesterol/HDL ratio	3.95 ± 6.02	3.55 ± 4.23	5.40 ± 16.76** ∧ ∧
Log_{10} (TG/ HDL-C), (AIP)	0.24	0.33	0.55

**represents $p < 0.05$ as compared to sea level, **represents $p < 0.01$ as compared to sea level, ***represents $p < 0.001$ as compared to sea level, ∧ represents $p < 0.05$ as compared to HA-D7, ∧∧ represents $p < 0.01$ as compared to HA-D7. AIP indicates Atherogenecity Index of Plasma.*

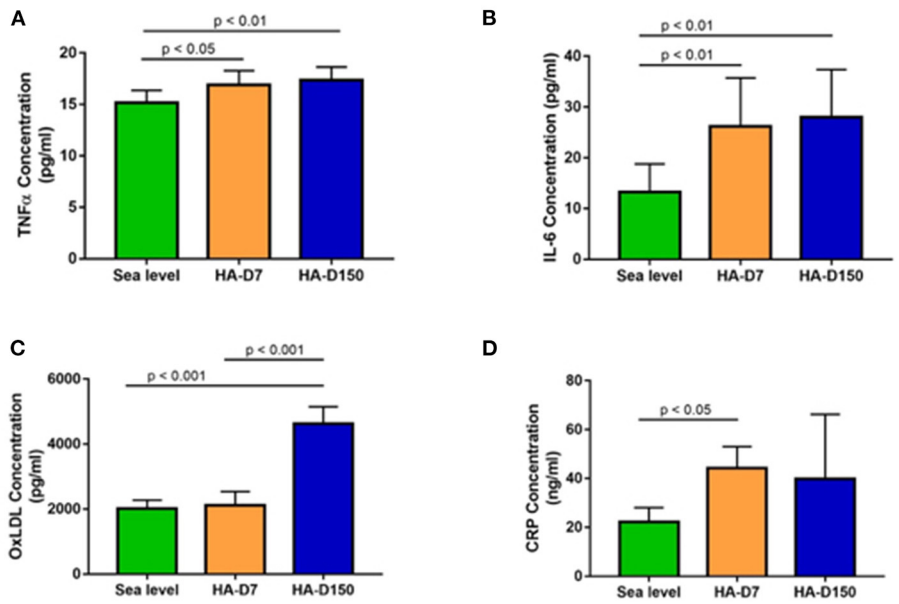

FIGURE 3 | Assessment of inflammatory cytokines in the study groups ($n = 8$). ELISA-based estimation of **(A)** TNF-α, **(B)** IL-6, **(C)** ox-LDL, and **(D)** CRP for sea level, HA-D7, and HA-D150 groups.

HA-D7 group. Persistent hypoxia at high altitude induces HIF-1α that upregulates stearoyl-CoA desaturase (SCD)-1 in the sterol regulatory element-binding protein (SREBP)-1c pathway resulting in increased hepatic *de novo* TGs synthesis (Siques et al., 2020). In contrast, we observed the lowest HDL levels for HA-D150. High altitude exposure compromises HDL maturation and alters the levels of HDL associated proteins limiting its protective functions (Gangwar et al., 2019). Consequently, ratios of LDL/HDL, cholesterol/HDL, and AIP, potential risk factors for coronary artery disease (CAD) were elevated for HA-D150. Both animal and human studies have reported that hypoxia increases plasma triglycerides by decreasing tissue uptake (Barnholt et al., 2006; Siques et al., 2014). Epidemiological studies have reported a high prevalence of hypercholesterolemia and low HDL levels for indigenous high land native populations of Peru (Mohanna et al., 2006), Chile (Santos et al., 2001), and Tibet (Sherpa et al., 2011). These studies along with our current observation suggest that extended stay at high altitude results in dyslipidemia and elevates proatherogenic lipoprotein levels.

There is accumulating evidence that HA exposure is associated with an inflammatory response and associated endothelial activation/dysfunction (Hartmann et al., 2000; Bruno et al., 2014; Boos et al., 2016) that aggravates many forms of cardiovascular diseases (Riley and Gavin, 2017; Parati et al., 2018). Healthy young lowlanders with no preexisting risk factors develop massive infarct and ischemic stroke during a prolonged stay at HA (above 4,270 m) (Jha et al., 2002). Acclimatized lowlanders exhibit increased levels of coronary risk factors after 15–18 months stay at the Indian trans-Himalayan Ladakh region (altitude more than 3,500 m) (Dhar et al., 2018). Hence, we measured proinflammatory cytokines IL-6, CRP, TNFα, and

oxidized LDL (oxLDL) levels in all three groups. The levels of IL-6 increased after altitude exposure (HA-D7) and levels were further increased after prolonged exposure (HA-D150). Studying cytokines after high altitude exposure, Hartmann et al. have reported increased IL-6 levels indicating considerable inflammation (Hartmann et al., 2000). Increased IL-6 level serves as an independent predictor of AMS (Boos et al., 2016) and plays a role in the pathogenesis of HAPE (Kubo et al., 1998). Similar higher levels of TNFα were observed after high altitude exposure and the highest levels were recorded for the HA-D150 group. Studying inflammatory cytokines in BAL fluid of HAPE patients, Kubo et al. have reported higher TNFα levels (Kubo et al., 1998) supporting our present observations. We also observed increased CRP levels for HA-D7 and the levels subsequently decreased for HA-D150 but remained higher than the sea level. Increased CRP levels have been reported for both acute (Hartmann et al., 2000; Gangwar et al., 2019) and prolonged high altitude exposure (Hu et al., 2016). Elevated levels of plasma CRP are associated with the risk of atherosclerotic events in general populations and show a predictive value even in terms of secondary prevention (Libby and Ridker, 2004; Calabro et al., 2009). Hence, we measured oxLDL levels that contribute to atherosclerotic plaque formation and progression by several mechanisms, including the induction of endothelial cell activation and dysfunction, macrophage foam cell formation, and smooth muscle cell migration and proliferation (Pirillo et al., 2013; Poznyak et al., 2020). We observed significantly higher oxLDL levels after a prolonged stay at high altitudes as compared to HA-D7 and sea level. Our present observations of elevated proinflammatory cytokines and oxLDL levels after prolonged exposure to high altitude indicate vascular inflammation leading to a proatherosclerotic state. A recent study evaluating long-term chronic intermittent hypobaric hypoxia-induced right ventricular hypertrophy has reported upregulation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a major OxLDL receptor on endothelial cell surface supports our current observations (Pena et al., 2020b).

We identified CA1 and CA2 as the topmost upregulated proteins for HA-D150, on average 2-fold higher than the HA-D7 group. These two are a member of the CA family that reversibly catalyzes the hydration of CO_2 to form HCO_3^-, which then rapidly binds to calcium ions to form calcium carbonate (Supuran, 2008). This well-known reaction is involved in a range of physiologic processes, ranging from CO_2 metabolism to cell proliferation and glucose/lipid metabolism (Gilmour, 2010). It is important to note that acetazolamide and other related CA inhibitors have been effectively used for the prevention and treatment of AMS and remain the standard of care for this indication (Swenson, 2006; Nieto Estrada et al., 2017). While there exists no difference between red blood cell CA activity between lowlanders and native highlanders (Gamboa et al., 2000), increased activity has been reported in patients with obstructive sleep apnea (OSA) (Hoff et al., 2020). Inhibition of CA activity curtails inflammation and experimental hypertension (Hudalla et al., 2019) suggesting that CAs are involved in the inflammation and vascular calcification (Adeva-Andany et al., 2015; Yuan et al., 2019). Though our results do not provide any direct evidence for CA-mediated vascular inflammation and calcification, their profound upregulation and existence of a proatherogenic propensity during long-term stay at high altitude suggests a possible link between CA and high altitude-induced dyslipidemia.

The present study is limited by studying only plasma samples and blood parameters for understanding the body response to hypoxia, whereas at organ and tissue level the response is more complex. All global plasma proteomics studies are constrained by a high dynamic range of plasma proteins, and even after several high abundance protein depletion steps, it is not possible to detect very low abundance proteins that may be of critical importance. We have studied only male volunteers for 3 months in the present study for specific reasons. A vast number of studies have reported sexual dimorphism for cardiovascular response and disorders particularly due to the sex hormone estrogen (Hester et al., 2019; Horiuchi et al., 2019; Hou et al., 2019; Shen et al., 2020; Ndzie Noah et al., 2021). It is important to note that estrogen levels widely vary between both the sex (Khosla et al., 1998), and cardiac adaptive responses significantly vary between males and females during chronic hypoxia (Bohuslavova et al., 2010). Hence to minimize variations, women were consciously excluded from the present study. However, such proteomics studies with women and longer durations of high altitude stay (6 months or more) could provide additional molecular information.

In summary, the present study reports elevated levels of SpO_2, HR, SBP, and DBP after long-term exposure to high altitude as compared to short-term exposure whereas the hemoglobin levels remained the same. Global plasma proteomics studies revealed upregulation of several apolipoproteins, and subsequent bioinformatics analysis revealed perturbation in assembly, remodeling, and clearance of plasma lipoproteins including VLDL and chylomicrons. In corroboration, we also observed higher TC, TGs, and LDL after a prolonged stay at high altitude indicating dyslipidemia. These observations were further supported by higher levels of inflammatory cytokines IL-6, TNFα, CRP as well as oxLDL. These cumulative results indicate persistent vascular inflammation after long-term exposure to high altitude leading to dyslipidemia and a proatherosclerotic condition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028070.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Defence Institute of Physiology and Allied Sciences (IEC/DIPAS/B2/1). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NK designed the experiments and wrote the manuscript with inputs from P. P and VS performed the experiments. RM, KR, UP, and NK collected samples at sea level and high altitude as well as recorded physiological parameters. P, RV, and NK analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.730601/full#supplementary-material

REFERENCES

Adeva-Andany, M. M., Fernandez-Fernandez, C., Sanchez-Bello, R., Donapetry-Garcia, C., and Martinez-Rodriguez, J. (2015). The role of carbonic anhydrase in the pathogenesis of vascular calcification in humans. *Atherosclerosis* 241, 183–191. doi: 10.1016/j.atherosclerosis.2015.05.012

Ahmad, Y., Sharma, N. K., Garg, I., Ahmad, M. F., Sharma, M., and Bhargava, K. (2013). An insight into the changes in human plasma proteome on adaptation to hypobaric hypoxia. *PLoS ONE* 8:e67548. doi: 10.1371/journal.pone.0067548

Ahmad, Y., Shukla, D., Garg, I., Sharma, N. K., Saxena, S., Malhotra, V. K., et al. (2011). Identification of haptoglobin and apolipoprotein A-I as biomarkers for high altitude pulmonary edema. *Funct. Integr. Genom.* 11, 407–417. doi: 10.1007/s10142-011-0234-3

Anand, A. C., Jha, S. K., Saha, A., Sharma, V., and Adya, C. M. (2001). Thrombosis as a complication of extended stay at high altitude. *Natl. Med. J. India* 14, 197–201.

Barnholt, K. E., Hoffman, A. R., Rock, P. B., Muza, S. R., Fulco, C. S., Braun, B., et al. (2006). Endocrine responses to acute and chronic high-altitude exposure (4,300 meters): modulating effects of caloric restriction. *Am. J. Physiol. Endocrinol. Metabol.* 290, E1078–1088. doi: 10.1152/ajpendo.00449.2005

Bartsch, P., and Gibbs, J. S. (2007). Effect of altitude on the heart and the lungs. *Circulation* 116, 2191–2202. doi: 10.1161/CIRCULATIONAHA.106.650796

Basnyat, B. (2018). The potential usefulness of serum biomarkers in high-altitude medicine. *J. Travel Med.* 25, 1–2. doi: 10.1093/jtm/tay083

Bohuslavova, R., Kolar, F., Kuthanova, L., Neckar, J., Tichopad, A., and Pavlinkova, G. (2010). Gene expression profiling of sex differences in HIF1-dependent adaptive cardiac responses to chronic hypoxia. *J. Appl. Physiol.* 109, 1195–1202. doi: 10.1152/japplphysiol.00366.2010

Boos, C. J., Woods, D. R., Varias, A., Biscocho, S., Heseltine, P., and Mellor, A. J. (2016). High altitude and acute mountain sickness and changes in circulating Endothelin-1, Interleukin-6, and Interleukin-17a. *High Alt. Med. Biol.* 17, 25–31. doi: 10.1089/ham.2015.0098

Bruno, R. M., Cogo, A., Ghiadoni, L., Duo, E., Pomidori, L., Sharma, R., et al. (2014). Cardiovascular function in healthy Himalayan high-altitude dwellers. *Atherosclerosis* 236, 47–53. doi: 10.1016/j.atherosclerosis.2014.06.017

Calabro, P., Golia, E., and Yeh, E. T. (2009). CRP and the risk of atherosclerotic events. *Semin. Immunopathol.* 31, 79–94. doi: 10.1007/s00281-009-0149-4

Calbet, J. A., Boushel, R., Radegran, G., Sondergaard, H., Wagner, P. D., and Saltin, B. (2003). Why is VO2 max after altitude acclimatization still reduced despite normalization of arterial O2 content? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284, R304–R316. doi: 10.1152/ajpregu.00156.2002

Dhar, P., Sharma, V. K., Das, S. K., Barhwal, K., Hota, S. K., and Singh, S. B. (2018). Differential responses of autonomic function in sea level residents, acclimatized lowlanders at >3500m and Himalayan high altitude natives at >3500m: a cross-sectional study. *Respir. Physiol. Neurobiol.* 254, 40–48. doi: 10.1016/j.resp.2018.04.002

Gallagher, S. A., and Hackett, P. H. (2004). High-altitude illness. *Emerg. Med. Clin. North Am.* 22, 329–355. doi: 10.1016/j.emc.2004.02.001

Gamboa, J., Caceda, R., Gamboa, A., and Monge, C. C. (2000). Carbonic anhydrase activity in the red blood cells of sea level and high altitude natives. *Biol. Res.* 33, 207–208. doi: 10.4067/S0716-97602000000300006

Gangwar, A., Pooja., Sharma, M., Singh, K., Patyal, A., Bhaumik, G., et al. (2019). Intermittent normobaric hypoxia facilitates high altitude acclimatization by curtailing hypoxia-induced inflammation and dyslipidemia. *Pflugers Arch.* 471, 949–959. doi: 10.1007/s00424-019-02273-4

Geyer, P. E., Kulak, N. A., Pichler, G., Holdt, L. M., Teupser, D., and Mann, M. (2016). Plasma proteome profiling to assess human health and disease. *Cell Syst.* 2, 185–195. doi: 10.1016/j.cels.2016.02.015

Gilmour, K. M. (2010). Perspectives on carbonic anhydrase. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 157, 193–197. doi: 10.1016/j.cbpa.2010.06.161

Gupta, N., and Ashraf, M. Z. (2012). Exposure to high altitude: a risk factor for venous thromboembolism? *Semin. Thromb. Hemost.* 38, 156–163. doi: 10.1055/s-0032-1301413

Hackett, P. H., and Roach, R. C. (2001). High-altitude illness. *N. Engl. J. Med.* 345, 107–114. doi: 10.1056/NEJM200107123450206

Hainsworth, R., Drinkhill, M. J., and Rivera-Chira, M. (2007). The autonomic nervous system at high altitude. *Clin. Auton. Res.* 17, 13–19. doi: 10.1007/s10286-006-0395-7

Hartmann, G., Tschop, M., Fischer, R., Bidlingmaier, C., Riepl, R., Tschop, K., et al. (2000). High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. *Cytokine* 12, 246–252. doi: 10.1006/cyto.1999.0533

Hester, J., Ventetuolo, C., and Lahm, T. (2019). Sex, gender, and sex hormones in pulmonary hypertension and right ventricular failure. *Compr. Physiol.* 10, 125–170. doi: 10.1002/cphy.c190011

Himadri, P., Kumari, S. S., Chitharanjan, M., and Dhananjay, S. (2010). Role of oxidative stress and inflammation in hypoxia-induced cerebral edema: a molecular approach. *High Alt. Med. Biol.* 11, 231–244. doi: 10.1089/ham.2009.1057

Hoff, E., Zou, D., Schiza, S., Demir, Y., Grote, L., Bouloukaki, I., et al. (2020). Carbonic anhydrase, obstructive sleep apnea and hypertension: effects of intervention. *J. Sleep Res.* 29:e12956. doi: 10.1111/jsr.12956

Horiuchi, M., Kirihara, Y., Fukuoka, Y., and Pontzer, H. (2019). Sex differences in respiratory and circulatory cost during hypoxic walking: potential impact on oxygen saturation. *Sci. Rep.* 9:9550. doi: 10.1038/s41598-019-44844-6

Hou, Y. P., Wu, J. L., Tan, C., Chen, Y., Guo, R., and Luo, Y. J. (2019). Sex-based differences in the prevalence of acute mountain sickness: a meta-analysis. *Mil. Med. Res.* 6:38. doi: 10.1186/s40779-019-0228-3

Houston, C. S., and Riley, R. L. (1947). Respiratory and circulatory changes during acclimatization to high altitude. *Am. J. Physiol.* 149, 565–588. doi: 10.1152/ajplegacy.1947.149.3.565

Hu, S. L., Xiong, W., Dai, Z. Q., Zhao, H. L., and Feng, H. (2016). Cognitive changes during prolonged stay at high altitude and its correlation with C-reactive protein. *PLoS ONE* 11:e0146290. doi: 10.1371/journal.pone.0146290

Hudalla, H., Michael, Z., Christodoulou, N., Willis, G. R., Fernandez-Gonzalez, A., Filatava, E. J., et al. (2019). Carbonic anhydrase inhibition ameliorates inflammation and experimental pulmonary hypertension. *Am. J. Respir. Cell Mol. Biol.* 61, 512–524. doi: 10.1165/rcmb.2018-0232OC

Ignjatovic, V., Geyer, P. E., Palaniappan, K. K., Chaaban, J. E., Omenn, G. S., Baker, M. S., et al. (2019). Mass spectrometry-based plasma proteomics: considerations from sample collection to achieving translational data. *J. Proteome Res*. 18, 4085–4097. doi: 10.1021/acs.jproteome.9b00503

Jha, S. K., Anand, A. C., Sharma, V., Kumar, N., and Adya, C. M. (2002). Stroke at high altitude: Indian experience. *High Alt. Med. Biol*. 3, 21–27. doi: 10.1089/152702902753639513

Julian, C. G., Subudhi, A. W., Hill, R. C., Wilson, M. J., Dimmen, A. C., Hansen, K. C., et al. (2014). Exploratory proteomic analysis of hypobaric hypoxia and acute mountain sickness in humans. *J. Appl. Physiol*. 116, 937–944. doi: 10.1152/japplphysiol.00362.2013

Khosla, S., Melton, L. J. 3rd, Atkinson, E. J., O'Fallon, W. M., Klee, G. G., and Riggs, B. L. (1998). Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. *J. Clin. Endocrinol. Metabol*. 83, 2266–2274. doi: 10.1210/jc.83.7.2266

Kubo, K., Hanaoka, M., Hayano, T., Miyahara, T., Hachiya, T., Hayasaka, M., et al. (1998). Inflammatory cytokines in BAL fluid and pulmonary hemodynamics in high-altitude pulmonary edema. *Respir. Physiol*. 111, 301–310. doi: 10.1016/S0034-5687(98)00006-1

Levett, D. Z., Fernandez, B. O., Riley, H. L., Martin, D. S., Mitchell, K., Leckstrom, C. A., et al. (2011). The role of nitrogen oxides in human adaptation to hypoxia. *Sci. Rep*. 1:109. doi: 10.1038/srep00109

Levett, D. Z., Vigano, A., Capitanio, D., Vasso, M., De Palma, S., Moriggi, M., et al. (2015). Changes in muscle proteomics in the course of the Caudwell Research Expedition to Mt. *Everest. Proteomics* 15, 160–171. doi: 10.1002/pmic.201400306

Libby, P., and Ridker, P. M. (2004). Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. *Am. J. Med.* 116(Suppl. 6A), 9S−16S. doi: 10.1016/j.amjmed.2004.02.006

Lu, H., Wang, R., Li, W., Xie, H., Wang, C., Hao, Y., et al. (2018). Plasma proteomic study of acute mountain sickness susceptible and resistant individuals. *Sci. Rep*. 8:1265. doi: 10.1038/s41598-018-19818-9

Mallet, R. T., Burtscher, J., Richalet, J. P., Millet, G. P., and Burtscher, M. (2021). Impact of high altitude on cardiovascular health: current perspectives. *Vasc. Health Risk Manag*. 17, 317–335. doi: 10.2147/VHRM.S294121

Mandolesi, G., Avancini, G., Bartesaghi, M., Bernardi, E., Pomidori, L., and Cogo, A. (2014). Long-term monitoring of oxygen saturation at altitude can be useful in predicting the subsequent development of moderate-to-severe acute mountain sickness. *Wilderness Environ. Med*. 25, 384–391. doi: 10.1016/j.wem.2014.04.015

Mohanna, S., Baracco, R., and Seclen, S. (2006). Lipid profile, waist circumference, and body mass index in a high altitude population. *High Alt. Med. Biol*. 7, 245–255. doi: 10.1089/ham.2006.7.245

Moore, L. G. (2001). Human genetic adaptation to high altitude. *High Alt. Med. Biol*. 2, 257–279. doi: 10.1089/152702901750265341

Murray, A. J. (2016). Energy metabolism and the high-altitude environment. *Exp. Physiol*. 101, 23–27. doi: 10.1113/EP085317

Murray, A. J., Montgomery, H. E., Feelisch, M., Grocott, M. P. W., and Martin, D. S. (2018). Metabolic adjustment to high-altitude hypoxia: from genetic signals to physiological implications. *Biochem. Soc. Trans*. 46, 599–607. doi: 10.1042/BST20170502

Muza, S. R., Beidleman, B. A., and Fulco, C. S. (2010). Altitude preexposure recommendations for inducing acclimatization. *High Alt. Med. Biol*. 11:87–92. doi: 10.1089/ham.2010.1006

Naeije, R. (2010). Physiological adaptation of the cardiovascular system to high altitude. *Prog. Cardiovasc. Dis*. 52, 456–466. doi: 10.1016/j.pcad.2010.03.004

Natarajan, P., Kohli, P., Baber, U., Nguyen, K. H., Sartori, S., Reilly, D. F., et al. (2015). Association of APOC3 loss-of-function mutations with plasma lipids and subclinical atherosclerosis: the multi-ethnic bioimage study. *J. Am. College Cardiol*. 66, 2053–2055. doi: 10.1016/j.jacc.2015.08.866

Ndzie Noah, M. L., Adzika, G. K., Mprah, R., Adekunle, A. O., Adu-Amankwaah, J., and Sun, H. (2021). Sex-gender disparities in cardiovascular diseases: the effects of estrogen on eNOS, lipid profile, and NFATs during catecholamine stress. *Front. Cardiovasc. Med*. 8:639946. doi: 10.3389/fcvm.2021.639946

Nieto Estrada, V. H., Molano Franco, D., Medina, R. D., Gonzalez Garay, A. G., Marti-Carvajal, A. J., and Arevalo-Rodriguez, I. (2017). Interventions for preventing high altitude illness: Part 1. Commonly-used classes of drugs. *Cochrane Database Syst. Rev*. 6:CD009761. doi: 10.1002/14651858.CD009761.pub2

Padhy, G., Gangwar, A., Sharma, M., Himashree, G., Singh, K., Bhaumik, G., et al. (2016). Plasma kallikrein-bradykinin pathway promotes circulatory nitric oxide metabolite availability during hypoxia. *Nitric Oxide* 55–56, 36–44. doi: 10.1016/j.niox.2016.02.009

Palmer, B. F. (2010). Physiology and pathophysiology with ascent to altitude. *Am. J. Med. Sci*. 340, 69–77. doi: 10.1097/MAJ.0b013e3181d3cdbe

Parati, G., Agostoni, P., Basnyat, B., Bilo, G., Brugger, H., Coca, A., et al. (2018). Clinical recommendations for high altitude exposure of individuals with pre-existing cardiovascular conditions: a joint statement by the European Society of Cardiology, the Council on Hypertension of the European Society of Cardiology, the European Society of Hypertension, the International Society of Mountain Medicine, the Italian Society of Hypertension and the Italian Society of Mountain Medicine. *Eur. Heart J*. 39, 1546–1554. doi: 10.1093/eurheartj/ehx720

Pena, E., Brito, J., El Alam, S., and Siques, P. (2020a). oxidative stress, kinase activity and inflammatory implications in right ventricular hypertrophy and heart failure under hypobaric hypoxia. *Int. J. Mol. Sci*. 21:6421. doi: 10.3390/ijms21176421

Pena, E., Siques, P., Brito, J., Arribas, S. M., Boger, R., Hannemann, J., et al. (2020b). Nox2 upregulation and p38alpha MAPK activation in right ventricular hypertrophy of rats exposed to long-term chronic intermittent hypobaric hypoxia. *Int. J. Mol. Sci*. 21:8576. doi: 10.3390/ijms21228576

Pernemalm, M., and Lehtio, J. (2014). Mass spectrometry-based plasma proteomics: state of the art and future outlook. *Expert Rev. Proteomics* 11, 431–448. doi: 10.1586/14789450.2014.901157

Pham, K., Parikh, K., and Heinrich, E. C. (2021). Hypoxia and inflammation: insights from high-altitude physiology. *Front. Physiol*. 12:676782. doi: 10.3389/fphys.2021.676782

Pirillo, A., Norata, G. D., and Catapano, A. L. (2013). LOX-1, OxLDL, and atherosclerosis. *Mediators Inflamm*. 2013:152786. doi: 10.1155/2013/152786

Pollin, T. I., Damcott, C. M., Shen, H., Ott, S. H., Shelton, J., Horenstein, R. B., et al. (2008). A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. *Science* 322, 1702–1705. doi: 10.1126/science.1161524

Pooja, S. M., Singh, K., Himashree, G., Bhaumik, G., Kumar, B., et al. (2020). Estrogen receptor (ESR1 and ESR2)-mediated activation of eNOS-NO-cGMP pathway facilitates high altitude acclimatization. *Nitric Oxide* 102, 12–20. doi: 10.1016/j.niox.2020.05.003

Poznyak, A. V., Nikiforov, N. G., Markin, A. M., Kashirskikh, D. A., Myasoedova, V. A., Gerasimova, E. V., et al. (2020). Overview of OxLDL and its impact on cardiovascular health: focus on atherosclerosis. *Front. Pharmacol*. 11:613780. doi: 10.3389/fphar.2020.613780

Reiner, Z. (2017). Hypertriglyceridaemia and risk of coronary artery disease. *Nat. Rev. Cardiol*. 14, 401–411. doi: 10.1038/nrcardio.2017.31

Reynafarje, C., Lozano, R., and Valdivieso, J. (1959). The polycythemia of high altitudes: iron metabolism and related aspects. *Blood* 14, 433–455. doi: 10.1182/blood.V14.4.433.433

Riley, C. J., and Gavin, M. (2017). Physiological changes to the cardiovascular system at high altitude and its effects on cardiovascular disease. *High Alt. Med. Biol*. 18, 102–113. doi: 10.1089/ham.2016.0112

Rocha, N. A., East, C., Zhang, J., and McCullough, P. A. (2017). ApoCIII as a cardiovascular risk factor and modulation by the novel lipid-lowering agent volanesorsen. *Curr. Atheroscler. Rep*. 19:62. doi: 10.1007/s11883-017-0697-3

Santos, J. L., Perez-Bravo, F., Carrasco, E., Calvillan, M., and Albala, C. (2001). Low prevalence of type 2 diabetes despite a high average body mass index in the Aymara natives from Chile. *Nutrition* 17, 305–309. doi: 10.1016/S0899-9007(00)00551-7

Sarada, S., Himadri, P., Mishra, C., Geetali, P., Ram, M. S., and Ilavazhagan, G. (2008). Role of oxidative stress and NFkB in hypoxia-induced pulmonary edema. *Exp. Biol. Med*. 233, 1088–1098. doi: 10.3181/0712-RM-337

Shen, Y., Yang, Y. Q., Liu, C., Yang, J., Zhang, J. H., Jin, J., et al. (2020). Association between physiological responses after exercise at low altitude and acute mountain sickness upon ascent is sex-dependent. *Mil. Med. Res*. 7:53. doi: 10.1186/s40779-020-00283-3

Sherpa, L. Y., Deji, S. H., Chongsuvivatwong, V., Luobu, O., Thelle, D. S., et al. (2011). Lipid profile and its association with risk factors for coronary heart disease in the highlanders of Lhasa, Tibet. *High Alt. Med. Biol.* 12, 57–63. doi: 10.1089/ham.2010.1050

Siervo, M., Riley, H. L., Fernandez, B. O., Leckstrom, C. A., Martin, D. S., Mitchell, K., et al. (2014). Effects of prolonged exposure to hypobaric hypoxia on oxidative stress, inflammation and gluco-insular regulation: the not-so-sweet price for good regulation. *PLoS ONE* 9:e94915. doi: 10.1371/journal.pone.0094915

Siques, P., Brito, J., Naveas, N., Pulido, R., De la Cruz, J. J., Mamani, M., et al. (2014). Plasma and liver lipid profiles in rats exposed to chronic hypobaric hypoxia: changes in metabolic pathways. *High Alt. Med. Biol.* 15:388–395. doi: 10.1089/ham.2013.1134

Siques, P., Brito, J., Ordenes, S., and Pena, E. (2020). Involvement of overweight and lipid metabolism in the development of pulmonary hypertension under conditions of chronic intermittent hypoxia. *Pulmonary Circ.* 10, 42–49. doi: 10.1177/2045894020930626

Soria, R., Egger, M., Scherrer, U., Bender, N., and Rimoldi, S. F. (2016). Pulmonary artery pressure and arterial oxygen saturation in people living at high or low altitude: systematic review and meta-analysis. *J. Appl. Physiol.* 121, 1151–1159. doi: 10.1152/japplphysiol.00394.2016

Supuran, C. T. (2008). Carbonic anhydrases–an overview. *Curr. Pharm. Des.* 14, 603–614. doi: 10.2174/138161208783877884

Swenson, E. R. (2006). Carbonic anhydrase inhibitors and hypoxic pulmonary vasoconstriction. *Respir. Physiol. Neurobiol.* 151, 209–216. doi: 10.1016/j.resp.2005.10.011

Tang, X. G., Wen, J., Zhang, X. S., and Jiang, D. C. (2018). Association between decreased osteopontin and acute mountain sickness upon rapid ascent to 3500 m among young Chinese men. *J. Travel Med.* 25, 1–6. doi: 10.1093/jtm/tay075

Tg, Hdl Working Group of the Exome Sequencing Project, N.H.L., Blood, I., Crosby, J., Peloso, G. M., Auer, P. L., et al. (2014). Loss-of-function mutations in APOC3, triglycerides, and coronary disease. *N. Engl. J. Med.* 371, 22–31. doi: 10.1056/NEJMoa1307095

Vats, P., Ray, K., Majumadar, D., Amitabh, J. D. A., Bayen, S., Akunov, A., et al. (2013). Changes in cardiovascular functions, lipid profile, and body composition at high altitude in two different ethnic groups. *High Alt. Med. Biol.* 14, 45–52. doi: 10.1089/ham.2012.1071

Virues-Ortega, J., Hogan, A. M., Baya-Botti, A., Kirkham, F. J., Baldeweg, T., Mahillo-Fernandez, I., et al. (2009). Survival and mortality in older adults living at high altitude in Bolivia: a preliminary report. *J. Am. Geriatr. Soc.* 57, 1955–1956. doi: 10.1111/j.1532-5415.2009.02468.x

Wang, Z., Liu, F., Ye, S., Jiang, P., Yu, X., Xu, J., et al. (2019). Plasma proteome profiling of high-altitude polycythemia using TMT-based quantitative proteomics approach. *J. Proteomics* 194, 60–69. doi: 10.1016/j.jprot.2018.12.031

Westerterp, M., Berbée, J. F. P., Pires, N. M. M., Mierlo, G. J. D., v., Kleemann, R., et al. (2007). Apolipoprotein C-I Is crucially involved in lipopolysaccharide-induced atherosclerosis development in apolipoprotein E-Knockout Mice. *Circulation* 116, 2173–2181. doi: 10.1161/CIRCULATIONAHA.107.693382

Yang, Y., Ma, L., Guan, W., Wang, Y., Du, Y., Ga, Q., et al. (2014). Differential plasma proteome analysis in patients with high-altitude pulmonary edema at the acute and recovery phases. *Exp. Ther. Med.* 7, 1160–1166. doi: 10.3892/etm.2014.1548

Yuan, L., Wang, M., Liu, T., Lei, Y., Miao, Q., Li, Q., et al. (2019). Carbonic anhydrase 1-mediated calcification is associated with atherosclerosis, and methazolamide alleviates its pathogenesis. *Front. Pharmacol.* 10:766. doi: 10.3389/fphar.2019.00766

Zhang, P., Li, Z., Yang, F., Ji, L., Yang, Y., Liu, C., et al. (2021). Novel insights into plasma biomarker candidates in patients with chronic mountain sickness based on proteomics. *Biosci. Rep.* 41:BSR20202219. doi: 10.1042/BSR20202219

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Hematological Parameters, Lipid Profile, and Cardiovascular Risk Analysis Among Genotype-Controlled Indigenous Kiwcha Men and Women Living at Low and High Altitudes

Esteban Ortiz-Prado[1,2], David Portilla[1], Johanna Mosquera-Moscoso[1], Katherine Simbaña-Rivera[1], Diego Duta[3], Israel Ochoa[4], German Burgos[5], Juan S. Izquierdo-Condoy[1], Eduardo Vásconez[1], Manuel Calvopiña[1] and Ginés Viscor[2]*

[1] One Health Research Group, Faculty of Medicine, Universidad de las Americas, Quito, Ecuador, [2] Department of Cell Biology, Physiology and Immunology, Universidad de Barcelona, Barcelona, Spain, [3] General Ward, Limoncocha Community Health Unit, Limoncocha, Ecuador, [4] General Ward, Oyacachi Community Health Unit, Oyacachi, Ecuador, [5] Faculty of Medicine, Universidad de Las Americas, Quito, Ecuador

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Simona Mrakic-Sposta,
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Danilo Bondi,
University of Studies G. d'Annunzio Chieti and Pescara, Italy
Gregoire P. Millet,
University of Lausanne, Switzerland

***Correspondence:**
Esteban Ortiz-Prado
e.ortizprado@gmail.com

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Introduction: Human adaptation to high altitude is due to characteristic adjustments at every physiological level. Differences in lipid profile and cardiovascular risk factors in altitude dwellers have been previously explored. Nevertheless, there are no reports available on genotype-controlled matches among different altitude-adapted indigenous populations.

Objective: To explore the possible differences in plasma lipid profile and cardiovascular risk among autochthonous Kiwcha people inhabitants of low and high-altitude locations.

Methodology: A cross-sectional analysis of plasmatic lipid profiles and cardiovascular risk factors in lowland Kiwchas from Limoncocha (230 m) and high-altitude Kiwchas from Oyacachi (3,800 m).

Results: In the low altitude group, 66% were women ($n = 78$) and 34% ($n = 40$) were men, whereas in the high altitude group, 59% ($n = 56$) were women and 41% ($n = 41\%$) were men. We found the proportion of overweight and obese individuals to be higher among low altitude dwellers ($p < 0.05$). Red blood cells (RBCs), hemoglobin concentration, and SpO_2% were higher among high altitude dwellers and the erythrocyte size was found to be smaller at high altitude. The group located at low altitude also showed lower levels of plasma cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), but most of these differences are not influenced by gender or elevation.

Conclusions: Living at an altitude elicits well-known adaptive physiological changes such as erythrocyte count, hemoglobin concentration, hematocrit level, and serum glucose level. We also report clinical differences in the plasma lipid profile, with higher levels of cholesterol, HDL, and LDL in inhabitants of the Andes

Mountain vs. their Amazonian basin peers. Despite this, we did not find significant differences in cardiovascular risk.

Keywords: high altitude, hypoxia, hematological profile, adaptation, lipid profile, cardiovascular risk

INTRODUCTION

Humans have developed adaptive mechanisms that allow them to live under extreme conditions. These conditions include cold and harsh environments such as those found at high-altitude locations. It has been difficult to define at which elevation the effects of high-altitude become more severe and where the threshold is located in terms of mild or severe hypoxia (West et al., 2007). Imray et al. (2011) used classification of high-altitude exposure in accordance with recommendations from the International Society of Mountain Medicine, a categorization that seems to be the most pragmatic (Imray et al., 2011). The author defined low altitude as everything that is located below 1,500 m, moderate or intermediate altitude from 1,500 to 2,500 m, high-altitude from 2,500 to 3,500 m, very high-altitude from 3,500 to 5,800 m, extreme high-altitude above 5,800 m, and death zone above 8,000 m (Imray et al., 2011).

Worldwide, more than 140 million people reside above 2,500 m (Pasha and Newman, 2010). Studying high-altitude dwellers is essential to understand the environmental, physiological, and genetic factors that are linked to the incidence and prevalence of different maladies in these populations (Miranda et al., 2019).

Acute and chronic exposure to high altitude has a variety of effects on human physiology and can be the cause of the occurrence of many diseases (Milledge, 2020). Barometric pressure decreases exponentially with increasing altitude. Consequently, the partial pressure of oxygen also decreases, despite which the composition of gases in the atmosphere remains unaltered. The physiological consequences of this reduction in oxygen availability begin to be noticeable, even at rest, from an altitude of 2,500 m (Ortiz-Prado et al., 2019). For that reason, residents at high altitudes have physiological and morphological adaptations that allow them to deal with these environmental conditions, whereas habitual residents at low altitudes must acclimatize once they ascend to these elevations (West, 2006). The anatomical, ventilatory, and cardiovascular differences between populations (residents at low vs. high altitudes) have been widely described; nevertheless, it is still unclear if those physiological alterations act as protective ("strain") or risk ("stress") factors (Sherpa et al., 2011; Ortiz-Prado et al., 2017; Dhiman et al., 2018).

One of the most controversial issues is the potentially higher cardiovascular risk among high altitude dwellers. The cardiovascular health of populations permanently living at high altitude may not only depend on the degree of altitude adaptation reached by this particular population but also on lifestyle factors and genetic predisposition (Aryal et al., 2015). In particular, various risk factors can be noticed among Andean highland populations including excessive erythrocytosis (Monge's disease or chronic mountain sickness) and a hypercoagulable-prothrombotic state linked to a higher incidence of thrombosis, probably due to venous blood flow stasis and secondary polycythemia (Zangari et al., 2013). On the other hand, factors such as hypercholesterolemia and hyperlipidemia seem to have a lower prevalence among highlanders, thus indicating a reduced risk of developing atherosclerosis and stroke (Faeh et al., 2009; Aryal et al., 2017; Ortiz-Prado et al., 2019).

Long-term exposure to hypobaric hypoxia seems to be linked to healthier blood lipid profiles when compared with those of residents living at sea level (Mohanna et al., 2006; Siqués et al., 2007; Vats et al., 2013). According to the report by Gonzales et al., who studied 158 people living at 4,100 m, the fraction of non-high-density lipoprotein (HDL) cholesterol and triglycerides is directly associated with the value of hemoglobin, and their increase, in turn, is associated with higher diastolic blood pressure. More specifically, high hemoglobin levels were directly associated with higher levels of total cholesterol, low-density lipoprotein (LDL), HDL, and triglycerides, and no association was found between hemoglobin and glucose (Gonzales and Tapia, 2013). Al Riyami et al. (2015) showed that altitude was the most significant factor affecting HDL-C, followed by gender, serum triglycerides, and finally the 2-h post prandial plasma glucose. Also, Vats et al. (2013) pointed out that in the process of acclimatization to high altitude, there is an increase in the diastolic blood pressure and heart rate, in addition to an increase in HDL levels. Although these responses have been described previously, the differences between the two indigenous groups, which shared the same ancestry but adapted to life at very different altitudes, have never been reported before. This fortunate circumstance gives us a great opportunity to understand the role of exposure to the altitude as a causal determinant of these differences disregarding genetic ancestry.

The objective of the current report is to explore the plasma lipid profile and cardiovascular risk differences among autochthonous Kiwcha populations permanently living at low and high altitudes.

METHODOLOGY

Study Design

A cross-sectional analysis of the differences in plasmatic lipid profiles and cardiovascular risk was carried out in two populations of Kiwcha natives from Ecuador living at two different elevations.

Abbreviations: LDL, Low-density lipoprotein; HDL, High-density lipoprotein; AHA, American Heart Association; HGDP-CEPH, Human Genome Diversity Project – Centre d'Étude du Polymorphisme Humain; EUR, European; AFR, African; NAM, Native American; AIMs, Ancestry Informative Markers; IQR, Interquartile Range; DS, Deviation Standard.

Setting
This study was carried out in Ecuador in two geographically different areas, the Andes mountain range and the Amazon Basin.

Participants
This study was carried out in 134 women and 79 men who voluntarily accepted to participate in the study. All the participants who voluntarily agreed are members of the Kiwcha indigenous group from Ecuador. The high-altitude group came from Oyacachi, a small Kiwcha community located at 3,800 m of elevation while the low-altitude group was the Kiwcha people living at Limoncocha, located at 230 m of elevation.

Inclusion Criteria
The study was conducted among healthy volunteers of both sexes without any type of comorbidity or chronic disease, between the ages of 18 and 85, who were born and currently residing in Oyacachi (high-altitude group), and in Limoncocha (low-altitude group).

Exclusion Criteria
Volunteers who were under 18 years of age, those who were born in another community, and those who do not habitually reside in the aforementioned parishes were excluded from the study.

Variables and Outcomes
Sociodemographic variables, such as age, sex, marital status, and place of residence were recorded. Vital signs were obtained by our team that included five doctors in the field. To assess arterial blood pressure, we used an upper arm blood pressure monitor 3 Series® Model: BP7100 from OMRON based on the American Heart Association (AHA) Recommendations for Blood Pressure Measurement (Smith, 2005). To evaluate body fat percentage, body mass index (BMI), and body weight we use the Omron Body Composition Monitor & Scale HBF-514C manufactured by OMRON which measures fat using the bioimpedance method. The temperature was measured using a portable Non-Contact Professional Medical Grade Infrared Thermometer. For the entire blood laboratory work, we included the following lipid profile serum parameters: LDL (mg/dl), HDL (mg/dl), triglycerides (mg/dl), and total cholesterol (mg/dl). We have also included mean fasting blood glucose levels (mg/dl) and clinical parameters including systolic and diastolic blood pressure, heart and respiratory rate, height and body weight, and BMI. We computed the 10-year risk of heart disease or stroke for ages between 40 and 79 years using the AHA risk calculator (http://Kiwcha.cvriskcalculator.com/). A blood sample was used to extract RNA to determine ancestry roots from both populations and confirm that they share the same genetic traits.

Outcome
The main outcome is to determine the different lipid profiles and cardiovascular risk ratios among genotype-matched Kiwcha indigenous people who live at high altitude vs. their counterparts who live at low altitude.

Data Sources
Individual-level sociodemographic information, place of residence, and past medical history were obtained *in situ* in both communities. A complete physical examination including measurement of body weight and height, arterial blood pressure, body temperature, resting heart, and respiratory rate, and arterial oxygen saturation was performed.

Study Size and Sample Size Calculation
In terms of the number of patients required to achieve significance, the sample size (n) and margin of error (E) were given by the following formula:

$$x = Z(^{c}/_{100})^{2} r(100 - r)$$

$$n = {}^{Nx}/_{[(N-1)E2+\,x]}$$

$$E = Sqrt[{}^{(N-\,n)x}/_{n(N-1)}]$$

where N is the population size ($n = 570$ in Oyacachi and $n = 890$ in Limoncocha), (r) is the fraction of expected responses (50%), and $Z(c/100)$ is the critical value for the confidence level (c). The total number of medical and physical evaluations required to achieve statistical significance was 82 for the high-altitude group and 96 for the low-altitude group. Through a nonprobability convenience-based sampling technique, 118 patients (40 men and 78 women) were included for Limoncocha and 95 patients were included for (39 men and 56 women) for Oyacachi.

Data Analysis
Descriptive statistics were used to analyze and visualize differences between the two populations. A chi-square test was performed to check the association or independence of categorical variables. When the expected values were <5 in any of the categories, Fisher's exact test or Spearman's test were used when the variable had evident asymmetries with histograms prior to the selection of the test. Additionally, a two-way ANOVA test was performed to determine the influence of gender and altitude of the populations on the continuous dependent variables, followed by age correction.

To compare the population ancestries for Oyachachi and Limoncocha, a t-test was performed, considering individual genotypes. Normal distribution and equal variance were assumed; the test concludes ($p = 0.05$) that there is no difference between any of the continental contributions of the three founding ethnic groups considered.

All statistical analyses accepted significance when p-value < 0.05. Calculations were completed using the IBM Corp. Released 2014. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: and R Core Team software 2018 version 3.5.1. Cartography was generated using QGIS Development Team 2.8 and all the references were managed using the open-source software Zotero 5.0.85.

DNA Extraction and Analysis of Ancestry Ratios

To compare the ancestry of the two populations, a subsample of 47 unrelated individuals (30 Oyacachi vs 17 Limoncocha) was selected. We looked for a subsample among all the individuals to identify those subjects who did not have any first-order degree of consanguinity, a condition that is based on our laboratory protocol for ancestry analysis. DNA extraction was performed from FTA cards (GE Healthcare) by the Chelex method. The extracts were then diluted to a concentration of 5 ng/ul using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA; Walsh et al., 1991). 46-plex autosomal ancestry informative deletion-insertion markers (46-plex AIMs-InDel) were amplified. Fluorescent amplicons were sized by capillary electrophoresis in Pop-7 polymer using a genetic analyzer ABI 3130 (Applied Biosystems, Austin, TX). Alleles were named by the software Genemapper V 3.1 (Life Technologies, Carlsbad, CA) following nomenclature described by Pereira et al. (2012). Taking into account trihybrid historic mixture in Ecuador (Santangelo et al., 2017; Toscanini et al., 2018; Zambrano et al., 2019), inference of ancestry proportions was obtained considering the admixture model with $K = 3$ (based on runs consisting of 100,000 burn-in steps, followed by 100,000 Markov Chain Monte Carlo (MCMC) using the STRUCTURE V2.3.4 software (Pritchard et al., 2000).

All runs were made without any prior information on the origin of samples and only considered the genetic background for the ancestral continental populations based on reference samples: European, EUR ($n = 158$); African, AFR ($n = 105$); and Native American, NAM ($n = 64$). Reference genotypes were extracted from the diversity panel of the Human Genome Diversity Project-Center d'Etude du Polymorphisme Humain (HGDP-CEPH). The populations selected as comparative groups for Africa were: Angola ($n = 1$), Botswana ($n = 4$), Central African Republic ($n = 23$), Congo ($n = 13$), Kenya ($n = 11$), Lesotho ($n = 1$), Namibia ($n = 6$), Nigeria ($n = 22$), Senegal ($n = 22$), and South Africa ($n = 2$); for South America: Brazil ($n = 22$), Colombia ($n = 7$), and Mexico ($n = 35$); and for Europe: France ($n = 52$), Italy ($n = 49$), Orkney Islands ($n = 15$), and Russia ($n = 42$).

Ethical Consideration

Full ethical approval was obtained (#MED.EOP.17.01) throughout the Universidad de las Americas bioethics committee (CEISH). All patients voluntarily signed informed consent. For people who could not read or write, an official community translator and a family member capable of understanding what was described in the document were used to explain the entire context of the project and ensure that there were no doubts about it. To protect the identity and autonomy of patients, all personal information was coded to ensure anonymity.

RESULTS

Demographic Results

A total of 213 subjects were recruited in both communities. 52.9% ($n = 118$) were included from the Limoncocha low altitude group and 47.1% ($n = 95$) from the Oyacachi high altitude group. In general, women represented 63% ($n = 134$) of the entire cohort and men 37% ($n = 79$).

Age and Sex Differences

In the low altitude group, 66% were women ($n = 78$) and 34% ($n = 40$) were men, whereas at high altitude, 59% ($n = 56$) were women and 41% ($n = 41\%$) were men (**Table 1**).

The median age for the low altitude group was 41 years and 36 years for men and women, respectively. The sex–age intergroup differences were not significant for all the groups (**Table 1**).

Weight and BMI Differences

In relation to weight, we found that women at low altitudes are on average 1.9 kilos lighter than women at high altitudes (60.84 kg ± 8.33 kg), but this difference was not statistically significant ($p = 0.374$). Men living at high altitudes are 20.7% lighter than their counterparts at low altitudes ($p < 0.0001$). We did not find any underweight adult subjects in any group; however, we found the proportion of overweight patients and those with obesity type I, II, and extreme obesity to be significantly higher among low altitude dwellers (**Table 1**).

Vital Signs Differences by Sex and Elevation

We found that arterial blood pressure tends to be higher in men (106/75 mmHg) than in women (102/70 mmHg). Nevertheless, this small difference is not significant. The Mean Arterial Blood Pressure (MAP) and systolic blood pressure were 6.2 and 7.5%, respectively, lower in men from the high-altitude group when compared to men from the low altitude group. These differences are statistically significant ($p = 0.01$ and 0.029) (**Table 2**).

In terms of heart rate frequency (beats per minute), high altitude dwellers have a 9.4% lower heart rate; nevertheless, gender and level of altitude did not influence heart rate calculated by a two-way ANOVA and the difference was not statistically significant ($p = 0.911$).

Despite this, we found a 5.2% lower peripheral blood oxygen saturation for the low altitude group. Gender and altitude did not influence SpO_2% calculated by a two-way ANOVA ($p = 0.076$) (**Table 2** and **Figure 1**).

Complete Blood Count (CBC), Biochemical Analysis, and Cardiovascular Risk Analysis Between Groups

Differences in white blood cell counts were not observed among the low and high-altitude groups (**Table 3**). For red blood cells (RBCs) count and microscopic features, we found that high altitude dwellers have higher cells counts and high levels of hematocrit and hemoglobin; however, they have smaller RBCs that contain less hemoglobin per erythrocyte. Nevertheless, after correcting for age, altitude, and sex, the differences did not reach the 5% established significant level (**Figure 2**).

In terms of serological biochemical parameters, we did not find significant differences in mean fasting blood glucose levels or lipid profiles. For instance, low altitude dwellers have significantly lower total cholesterol, lower HDL, and

TABLE 1 | Demographic characteristics, weight, height, and body mass index (BMI) of the two populations in relation to sex.

		Low altitude (230 m)	High altitude (3800 m)	(%) Diff.	*p*-value
Median age (IQR)	Men	42.0 (30.0–52.0)	36.0 (25.0–57.0)	15.4	0.137
	Women	41.0 (30.0–59.0)	36.0 (29.0–48.0)	13	
Young adult (18–35 Kiwcha)	Men	24 (54.5)	27 (67.5)	11.8	0.475
	Women	45 (57.0)	41 (73.2)	9.3	0.086
Adult (36–64 Kiwcha)	Men	15 (34.1)	10 (25.0)	40	0.475
	Women	19 (24.1)	11 (19.6)	53.3	0.086
Elderly (>65 Kiwcha)	Men	5 (11.4)	3 (7.5)	50	0.475
	Women	15 (19.0)	4 (7.1)	115.8	0.086
Weight (kg)	Men	74.2 ± 10.8	60.3 ± 8.71	20.7	**0.0001**
	Women	62.7 ± 14.4	60.8 ± 8.3	3.1	
Height (cm)	Men	159.9 ± 6.3	155.5 ± 9.93	2.8	**0.001**
	Women	149.2 ± 7.0	152.6 ± 8.6	2.3	
Normal weight (18.5–24.9)	Men	5 (12.5)	21 (53.8)	123.1	**0.001**
	Women	25 (32.1)	20 (35.7)	22.2	**0.036**
Overweight (25–29.9)	Men	22 (55.0)	16 (41.0)	31.6	**0.001**
	Women	31 (39.7)	29 (51.8)	6.67	**0.036**
Obesity type I and II (30–39.9)	Men	7 (17.5)	2 (5.1)	111.1	**0.001**
	Women	12 (15.4)	7 (12.5)	52.6	**0.036**
Extreme obesity (>40)	Men	6 (15.0)	0 (0.0)		**0.001**
	Women	10 (12.8)	0 (0.0)		**0.036**

Mean ± SD; IQR, interquartile range. Bold values are Statistically significant difference at 95% confidence level.

TABLE 2 | Description of the main vital signs of both populations including arterial blood pressure, heart and respiratory rate, temperature, and blood peripherical oxygen saturation.

Vital sign		Low altitude (230 m)	High altitude (3,800 m)	(%) Diff	Sig.
SBP	Women	100 (90–110)	104 (90–120)	3.9	0.029
	Men	110 (100–120)	102(99–110)	7.5	
DBP	Women	70.0 (60–80)	70 (70–80)	0	0.016
	Men	80 (70–80)	70 (60–80)	13.3	
MAP	Women	74.9 ± 8.7	77 ± 11	3	0.010
	Men	80.2 ± 8.3	75.3 ± 8.6	6.2	
HR' (m ±SD)	Women	73. ± 10	66.0 ± 11	10.1	0.911
	Men	71 ± 13	65 ± 9	8.8	
RR' (m ±SD)	Women	18 ± 18–19	18 ± 18–23	0	0.346
	Men	18 ± 18–22	18 ± 18–21	0	
SpO_2%	Women	98 ± 97–99	93 ± 91–94	5.2	0.076
	Men	98 ± 97–98	93 ± 92–94	5.2	
T°	Women	36.7 ± 36.3–37.2	36 ± 36–36	0.8	0.565
	Men	36.5 ± 36.3–37.2	36.1 ± 35.7–36.7	1.1	

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; HR', Heart Rate Beats per minute; RR', Respiratory rate breaths per minute; m ±SD, Mean ± standard deviation; IQR, interquartile range; SpO_2%, peripheral Blood Oxygen saturation; T°, Body Temperature; MAP, Mean Arterial Blood Pressure (mmHg).

lower LDL values for both sexes. Nevertheless, triglycerides are on average 26% lower among high altitude dwellers. Despite these clinical differences, after correcting for age, sex and altitude, they did not reach significance at 5% level (**Figure 3**).

When computing a 10-year risk of heart disease or stroke using the atherosclerotic cardiovascular disease (ASCVD) algorithm published in the 2013 American college of cardiology (ACC)/AHA Guideline on the Assessment of Cardiovascular Risk, we did not find any statistically significant difference between groups (**Table 3**).

Gender and the level of altitude did not influence the overall blood biochemical analysis calculated by the two-way ANOVA. However, the combination of gender and altitude showed to significantly affect mean corpuscular hemoglobin concentration ($p = 0.031$),

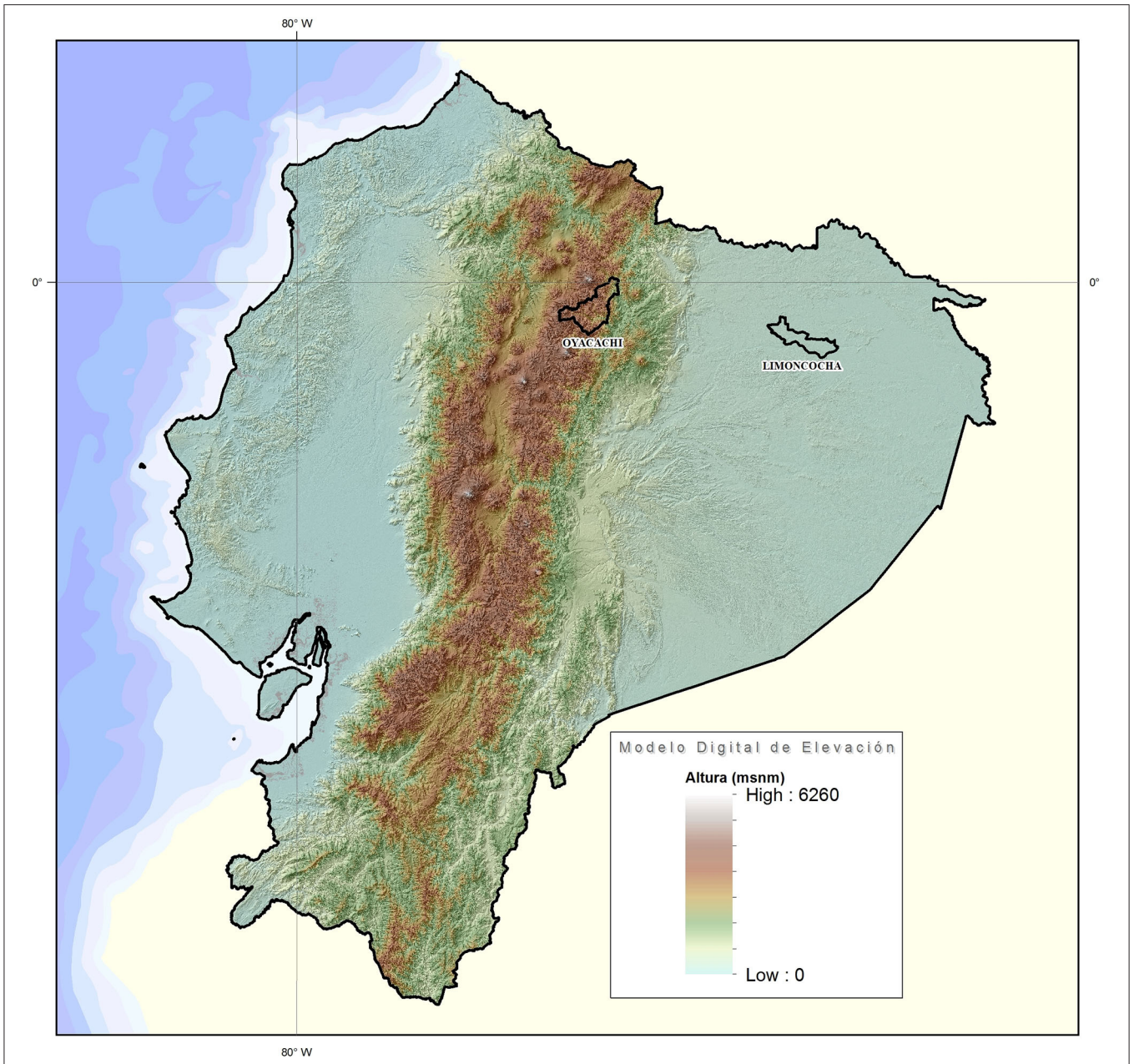

FIGURE 1 | Vital signs differences according to sex and altitude.

even when this relationship considers adjustment for age ($p = 0.033$).

Ancestry proportions between Oyacachi and Limoncocha are not statistically different. Both communities retain Native American ancestry >97% and vary slightly in the European (0.6 vs. 1.4%) and Afro (0.3 vs. 0.7%) contributions. However, they are highly conserved populations in general, Oyacachi being the most mixed, considering the data obtained from this sample and analysis (**Figure 4**).

DISCUSSION

Our study demonstrates that hematological, biochemical, and some clinical parameters differ between the two populations that share the same ancestral origin but have resided for centuries at two different geographical locations. Most of them are not influenced by gender or elevation. While all these differences have been described in different populations, this is the first time that we have been able to determine them as genetically controlled populations that share genetic, sociodemographic, and

TABLE 3 | Complete blood count (CBC) and blood biochemical analysis in low and high-altitude dwellers.

	Women		Men		
	Low altitude (230 m)	**High altitude (3,800 m)**	**Low altitude (230 m)**	**High altitude (3,800 m)**	***P*-value**
Lymphocytes	7.0 ± 2.0	6.0 ± 2.0	7.0 ± 2.0	6.0 ± 1.0	0.163
Neutrophiles	56.0 ± 8.0	55.0 ± 9.0	55.0 ± 7.0	52.0 ± 9.0	0.416
Lymphocytes	36.0 (32.0–42.0)	36.0 (31.0–40.0)	36.0 (32.0–40.0)	37.0 (32.0–46.0)	0.763
Monocytes	6.0 (5.0–7.0)	6.0 (5.0–7.0)	7.0 (6.0–9.0)	7.0 (6.0–8.0)	0.418
Eosinophiles	2.0 (1.0–2.0)	1.0 (1.0–3.0)	2.0 (2.0–3.0)	2.0 (1.0–3.0)	0.190
Hematocrit	41.0 (38.0–42.0)	47.0 (45.0–49.0)	45.0 (43.0–47.0)	52.0 (50.0–54.0)	0.515
Hemoglobin	13.45 ± 1.01	15.23 ± 1.10	15.31 ± 1.11	17.06 ± 1.01	0.897
RBC	4.0 ± 4.0–5.0	5.0 ± 5.0–5.0	5.6 ± 5.3–6.0	6.0 ± 5.0–6.5	0.363
Platelets	263.00 ± 52.00	276.00 ± 47.00	257.00 ± 53.00	257.00 ± 55.00	0.368
MCV	94.00 ± 4.00	92.00 ± 4.00	93.00 ± 3.00	91.00 ± 4.00	0.826
MCH	32.0 (30.0–32.0)	30.0 (29.0–31.0)	32.0 (31.0–32.0)	30.0 (29.0–31.0)	0.250
MCHC	33.0 (33.0–34.0)	33.0 (32.0–33.0)	34.0 (34.0–35.0)	33.0 (33.0–33.0)	**0.031**
Glucose	89 (83–95)	89 (84–92)	91.0 (83.0–97.0)	90.0 (84.0–95.0)	0.411
Cholesterol	174 ± 37	193.0 ± 28.0	167.0 ± 38.0	196.0 ±30.0	0.275
Triglycerides	127 (90–179)	90 (73–143)	133.0 (106.−180)	110.0 (78.0–146.0)	0.438
HDL	46.0 (40–55)	56.0 (46.0–71.0)	41.0 (38.0–47.0)	49.0 (44.0–60.0)	0.610
LDL	98.0 ± 32.0	113.0 ± 22.0	93.0 ± 34.0	117.0 ± 26.0	0.278
AHA Heart Risk	2.0 (1.0–5.0)	1.0 (1.0–5.0)	3.0 (2.0–5.0)	5.0 (2.0–9.0)	0.461

RBC, Red Blood Cells; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration. Bold value is statistically significant difference at 95% confidence level.

economical similarities, and geographically distinct territories (GAD Oyacachi, 2019; GAG Limoncocha, 2019).

Some of the differences that we have found, especially anthropometric distinctions are probably due to the adaptive processes. These processes have been described in several investigations that have explained how humans chronically exposed to high altitudes become more fit to function under hypoxic conditions (Julian and Moore, 2019).

The results of our study compare anthropometric differences in a genotype-controlled indigenous adult population living at low (230 m) and high altitudes (3,800 m). When analyzing the data, we observe that in general, women from high altitudes are slightly lighter and slightly taller than women from the lowlands (Merrill, 2020); nevertheless, men from high altitudes are significantly shorter and lighter than men from low altitudes. Our findings are similar to those reported in Bolivia by Leatherman et al. (1984). This study conducted an anthropometric survey among 138 men from rural mountainous areas of Bolivia (3,700 m) and concluded that men from high altitudes are shorter and lighter than their low altitude counterparts (Leatherman et al., 1984). Among Quechuas, a similar native group from Peru, Toselli et al. (2001) found individuals shorter at high altitudes in relationship to their corporal mass (Toselli et al., 2001). In contrast to earlier findings, however, no evidence of these results was detected by Khalid (1995) when they showed that high altitude residents from Saudi Arabia were significantly heavier and taller than the low altitude control group. These differences between two populations (the Andean and the Saudis) could demonstrate differences in terms of adaptation, something that has been described extensively before (Moore et al., 1998, 2011; Beall, 2007; Tyagi et al., 2008; Moore, 2017a).

On the other hand, women from high altitudes have a higher proportion of obesity than their low altitude counterparts, possibly due to cultural conditions that force women to stay at home cooking while men leave their houses to work (Khalid, 2007; Lin et al., 2018).

It has been hypothesized that at least 5% of high-altitude natives from Peru possess a newly discovered gene named *FBN1*. This gene seems to be associated with favoring high altitude Andean natives with low stature and possibly thicker skin (Pennisi, 2018). It is well-known that high altitude dwellers and animals are often smaller, an evolutionary response to the shortage of food or oxygen and thicker skin, which may help shield the body from intense UV radiation in such places (West, 2012; Pennisi, 2018).

It is well-known that weight among newborns is significantly lower among high-altitude neonates than their sea-level counterparts (Al-Shehri et al., 2005; Hoke and Leatherman, 2019), a situation that might continue not only during pregnancy but during the 1st years of childhood and adolescence (Lichty et al., 1957; Iannotti et al., 2009; Moore et al., 2011).

The fact that newborns are smaller has to do with an adaptive process that aims to reduce oxygen consumption by the fetus, being more efficient to deliver oxygen to a smaller organism through a smaller placenta (Krüger and Arias-Stella, 1970; Zamudio, 2003; Dolma et al., 2021).

Besides anthropometric differences, high altitude residents had superior lung capacities, enhanced vascularity, a blunted ventilatory response to sustained hypoxia and lower exercise

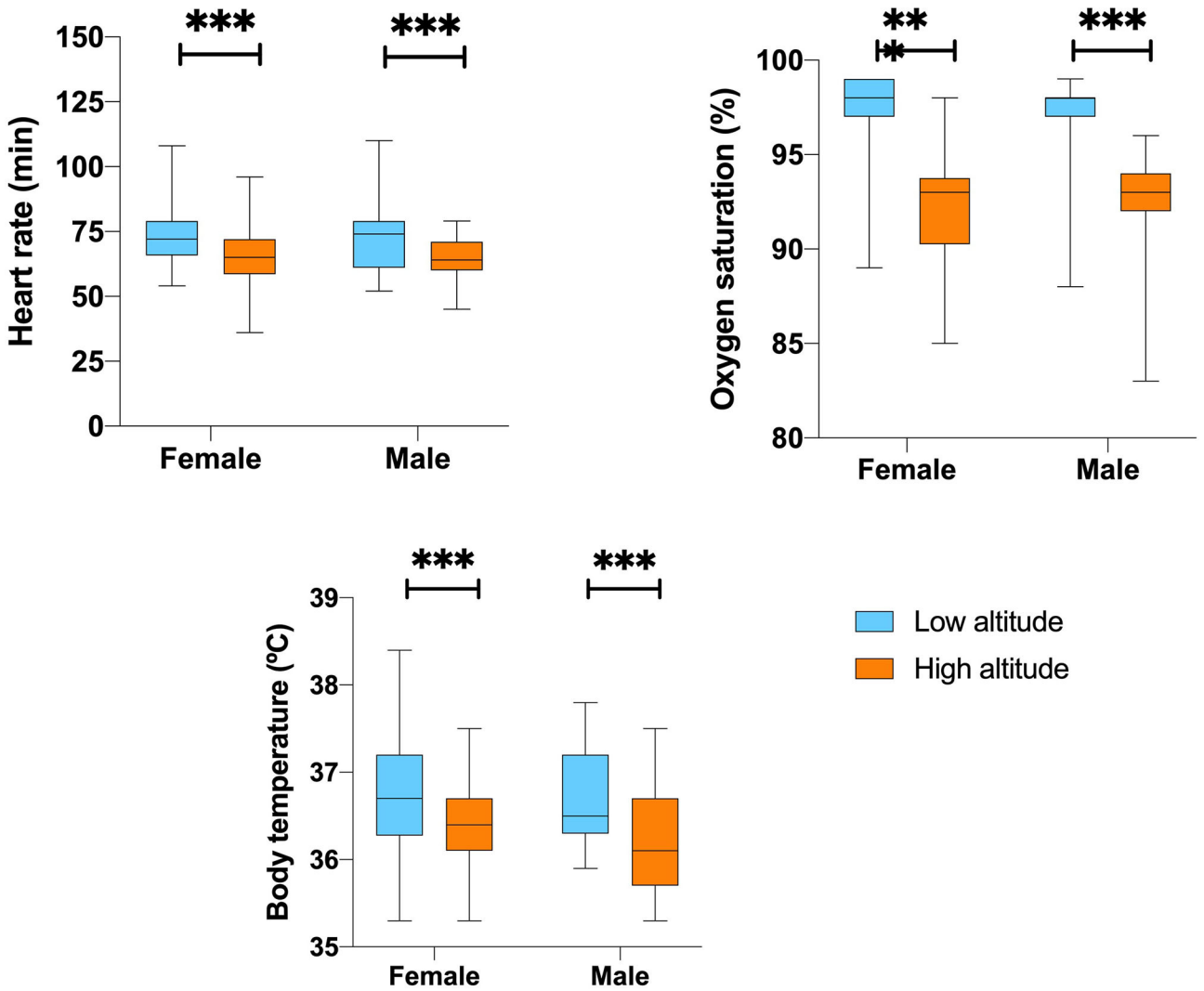

FIGURE 2 | The differences of Hematological parameter according to sex by altitude. **,***Represents outliers values.

ventilation, and overall superior efficiency of O_2 transport, utilization, and distribution (Zhuang et al., 1993; Brutsaert et al., 2005; Moore, 2017a; Ortiz-Prado et al., 2019). In the present study, we tried to identify whether there are physiological differences that are not necessarily due to sociocultural, social, economic, or differences in habit. According to the latest data from local governments in both Oyacachi and Limoncocha, the schooling rate, mortality rate, economic dependence, and access to health care services are similar in both parishes (GAD Oyacachi, 2019; GAG Limoncocha, 2019). Both parishes have only one health center provided regulated by the Ministry of Public Health (MoH).

In relation to high altitude lifestyle differences, habits, and endogenous preconditioning, gathering data is a complex task. Different populations have different eating habits, different lifestyles, and they usually subsist in a way different than their low-land counterparts (Westerterp, 2001; Lundby et al., 2006; Li and Zhao, 2015; Brutsaert, 2016). The data about risk factors available in Ecuador suggest that people living in provinces from the highlands consume more alcohol (17.1 vs. 9.1%) and smoke more (6.5 vs. 2.5%) than the people living at lower altitudes (Freire et al., 2015). In a general nationwide analysis, the National Institute of Statistics and Censuses of Ecuador (INEC) reported that people in the coast region seem to have a higher consumption of carbohydrates (36 vs. 30%) than those living in the highlands (INEC, 2018). Although these data on dietary variability could be extrapolated to the population living at high altitudes in Ecuador, it is well-known that people visiting high altitude locations have a significant loss in appetite and an accelerated metabolism that might speed up weight loss (Karl et al., 2018; Rausch et al., 2018).

The aforementioned similarities and some differences shared by both populations might not be fully responsible for our findings. We believe that physiological, hematological, and lipid

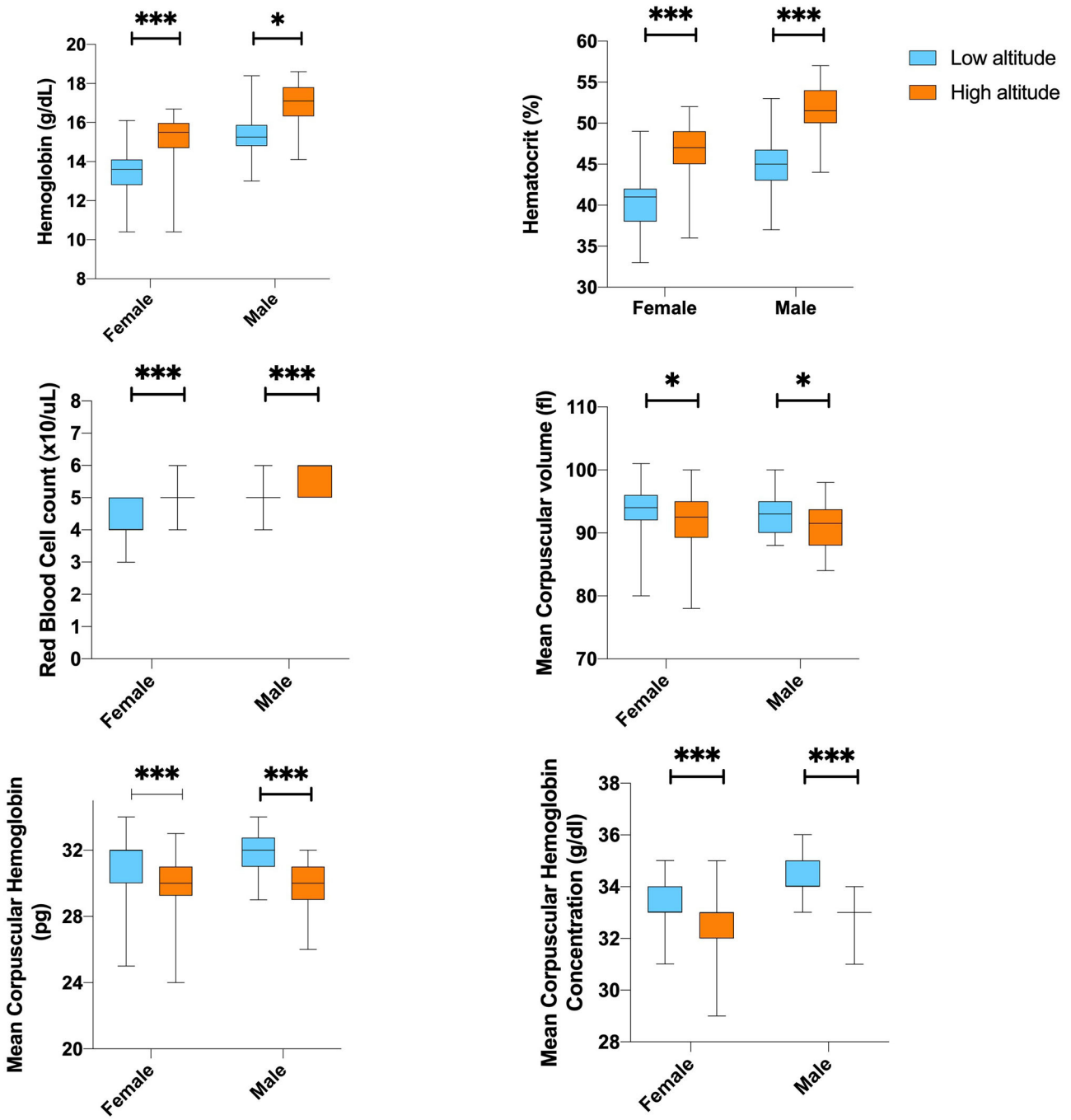

FIGURE 3 | Blood biochemistry and lipid profile difference value according to sex by altitude. **,***Represents outliers values.

profile differences have a genetic, respiratory, circulatory, and adaptive origin although most of them were not influenced by gender or elevation. For instance, we found that heart rate (HR') within the high-altitude population was 7 beats per minute slower than those at low altitudes and men always report lower MAP than women. This may be explained by the significantly high polycythemia described at high altitudes (Winslow, 1984). The higher the number of RBCs, the easier the oxygen transport, translating into a reduced cardiac output among adapted populations (West et al., 2007; Miggitsch et al., 2009). In a recently published analysis, Holmström et al., suggested that a lower metabolic rate and greater parasympathetic activity might be common among highlanders (Holmström et al., 2020).

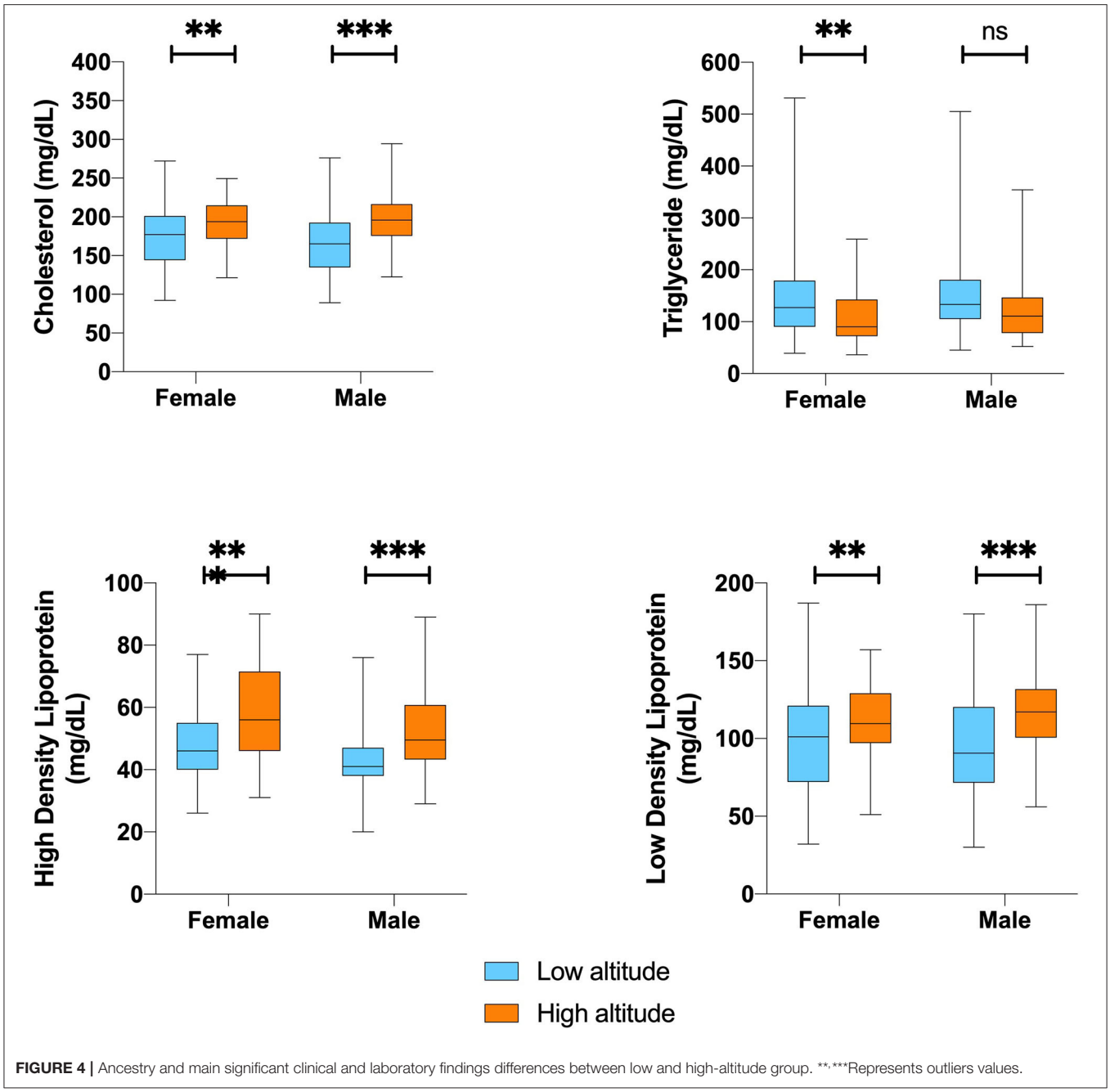

FIGURE 4 | Ancestry and main significant clinical and laboratory findings differences between low and high-altitude group. **,***Represents outliers values.

Having smaller RBCs, higher hemoglobin concentrations, lower MAP, and other differences might be, in part, attributed to their adaptational process experienced for centuries of living at different altitudes (Moore et al., 2011; Moore, 2017b). The Kiwcha population living at 230 m above sea level migrated further south centuries ago, while the Kiwcha group living above 3,800 m above sea level found a place to successfully thrive at a high altitude (Cardoso et al., 2012). When comparing the data obtained from both indigenous groups located at low and high altitudes, we did not find differences in the profile of their white blood cells; however, the size of RBCs and hemoglobin composition were found to be clinically different as expected and noted in several previous studies (Beall et al., 1998; Beall, 2006; Storz, 2007).

The difference in the number of RBCs and their size is expected since the low availability of oxygen at high altitudes due to the low barometric pressure causes a positive response on erythropoiesis and the subsequent production of RBCs (Zhong et al., 2015; Akunov et al., 2018; Ortiz-Prado et al., 2019).

The observed differences within the high-altitude population (**Figure 5**) might indicate an increased oxygen-carrying capacity (Samaja et al., 2003). The higher the production of the

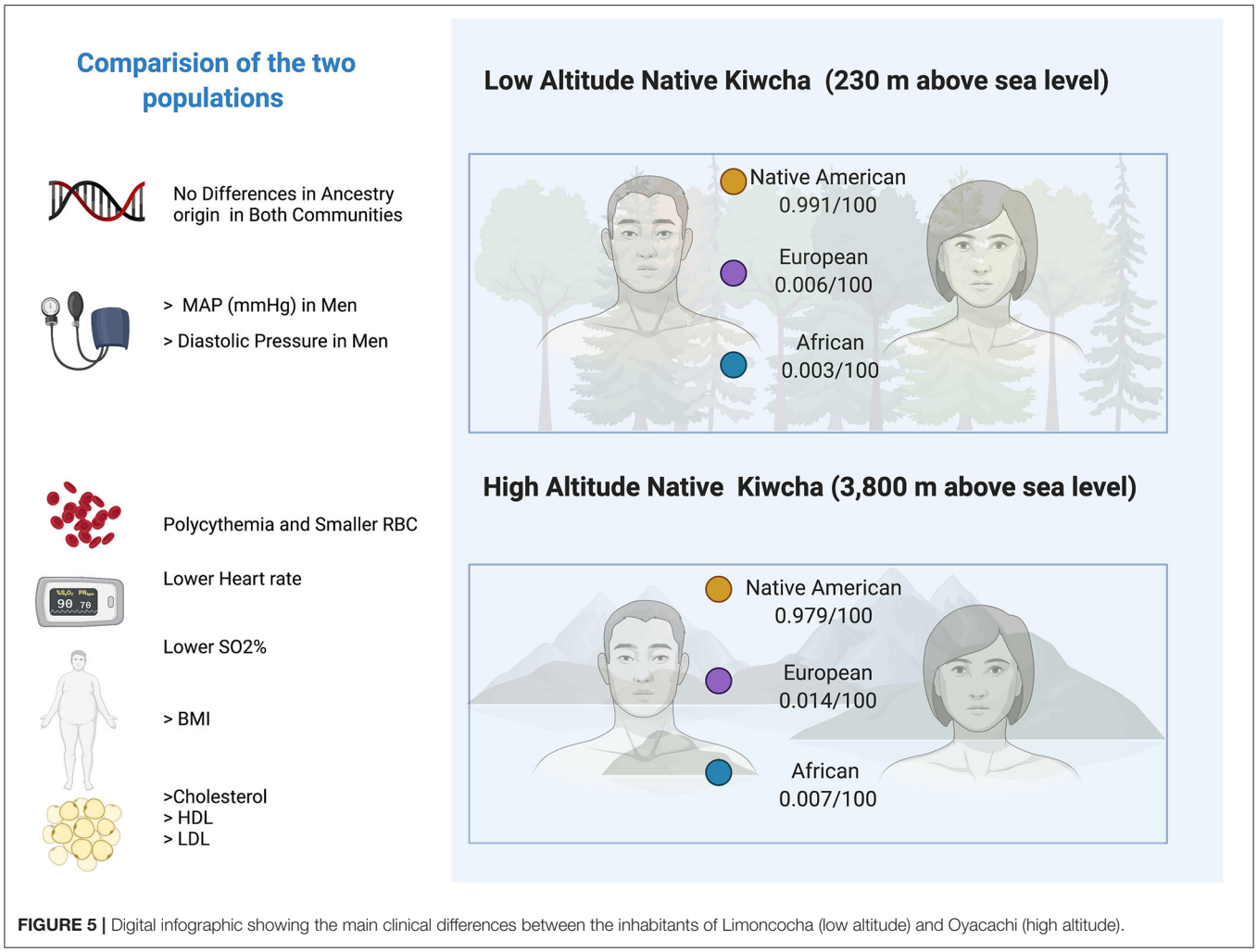

FIGURE 5 | Digital infographic showing the main clinical differences between the inhabitants of Limoncocha (low altitude) and Oyacachi (high altitude).

RBCs the thicker the blood, therefore, adaptative mechanisms based on a slightly reduced size of the RBCs and lower hemoglobin concentration within the erythrocyte might confer an evolutive advantage, reducing the risk of blood stasis (Stobdan et al., 2017).

Also noticeable are the differences in plasma lipid profile, as the group located at low altitude is more prone to having higher levels of triglycerides, especially among women, whereas the group located at a high altitude presents higher total cholesterol serum level and LDL and HDL levels which differ from men to women. Partially supporting these findings, high rates of hypercholesterolemia have been described in adult populations above 3,600 m that inhabit Peru and Tibet (Mohanna et al., 2006; Sherpa et al., 2011). However, the study of the influence of altitude on the lipid profile parameters has not been able to show causality due to the wide variability in the available data. A study by Ranhotra and Sharma, when comparing two populations of indigenous Khasis adults living at high and low altitudes, showed a decrease in total cholesterol and LDL of high-altitude residents, accompanied by a decrease in triglyceride levels at high altitudes (Ranhotra and Sharma, 2010). Similarly, a study by Siqués et al. carried out in natives of low altitudes who were exposed for 8 months at a height of 3,550 m did not reveal changes in total cholesterol levels and was accompanied by an increase in the concentration of triglycerides after altitude exposure (Siqués et al., 2007). Therefore, the influence of external factors such as physical activity, sedentary lifestyle, diet, and tobacco consumption has a considerable impact on the lipid profile of the altitude inhabitants.

These differences in habit patterns, lipid profile, and even in the ratio between obese and non-obese populations could be associated with lower mortality caused by cerebrovascular and cardiovascular diseases at high altitudes (Faeh et al., 2009; Burtscher et al., 2021).

Faeh et al. (2009) and Burtscher et al. (2021), provided data supporting the statement that living at a moderate altitude (1,000–2,000 m) elicits beneficial effects on all-cause mortality for both sexes, including diseases of the circulatory system in Switzerland and Austria, respectively (Faeh et al., 2009; Burtscher et al., 2021).

In the Kiwcha's case, the geographical isolation, and consequent sociodemographic and cultural factors that have been exposed over time, determine some behavioral differences

between the Kiwcha inhabits of Oyacachi and Limoncocha living at different altitudes, which also may have an influence on our findings (GAD Oyacachi, 2019; GAG Limoncocha, 2019).

Despite not having found significant differences in the risk for the development of heart disease and stroke, lower rates of coronary heart disease and stroke have been observed in the European population living at moderate altitudes (Faeh et al., 2009; Burtscher, 2016), and a progressive decrease in mortality from coronary heart disease and stroke has been observed as the altitude increases. External factors such as hypoxia level and solar radiation can also play a role. However, these effects are mainly observed at moderate elevations (around 2,000 m) in contrast to higher elevations (above 3,000 m; Moore, 2001; Faeh et al., 2009; Burtscher, 2016).

The study of these Andean populations confers an interesting opportunity to explore differences in a well-controlled group. The Oyacachi Kiwcha population (high altitude) and the Limoncocha group have evolved differently thanks to their geographical differences. In our context, having two populations that are genetically similar but have adapted to their landscapes for more than 500 years may provide important information on the mechanisms that could be linked to adaptation. As the adaptation to chronic hypoxia is polygenic, molecular adaptations may differ from those found in other parts of the planet, as has been seen among people living in the Himalayas or the mountainous areas of Ethiopia (Moore, 2001; Azad et al., 2017).

For instance, a recently published study suggests that both genetic predisposition and environmental exposure determine the size and function of human organs such as the spleen (Holmström et al., 2020). Although this information has not been compared with Andean natives, the increased spleen size found among Sherpas might also be linked to an improved circulating hemoglobin function (Holmström et al., 2020).

In an extensive literature review by Azad et al. (2017), the authors described the genomic implications of the adaptation of different organisms to high altitude (Azad et al., 2017). They described how a series of genetic components gave rise to the different bio-molecular pathways that regulate oxygen transport, the circulatory system functioning or the overall erythrocyte, oxygen, and hemoglobin homeostasis (Azad et al., 2017).

We suggest that several molecular and physiological mechanisms that have yet to be revealed might play a direct role in explaining some of the differences described in this study. Although numerous factors and variables could not be controlled, the reported findings provide new insights about an understudied population.

LIMITATIONS

The main limitation of this study was the absence of a dietary and exercise assessment, as diet massively alters blood lipid profile. Another limitation was that despite obtaining a significant sample size to carry out this study, not the entire population belonging to these indigenous communities that met the inclusion criteria was willing to participate. So, even if it is a small probability, it cannot rule out that the inclusion of the data corresponding to those people who did not participate could produce variations in our results or even alter our interpretation. Another potential weakness is the gender asymmetry in the sample size because men were a lower number of participants than women.

CONCLUSION

Permanent life at both altitudes induced well-known adaptive responses in Kiwcha dwellers: increased number of erythrocytes, hemoglobin concentration, hematocrit level, and serum glucose level. Although we have found remarkable differences in the plasma lipid profile between the populations at the two altitudes, these alterations did not seem to be influenced by altitude, sex, or age.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Universidad de las Americas, CEISH. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EO-P was fully responsible for the conceptualization, data collection, and elaboration of the study. EO-P, DP, and JM-M participated in drafting the manuscript equally and are fully responsible for it. DP, JM-M, and DD visited indigenous communities and collected samples. KS-R, JI-C, and EO-P contributed to the data collection and the construction of figures and tables. EO-P, MC, EV, JI-C, and GV contributed to the descriptive statistical analysis and the discussion section of the manuscript. GB was responsible for DNA extraction and analysis of ancestry ratios. EO-P critically reviewed the entire manuscript and produced several comments prior to submission. All the authors have read and approved the final version of the manuscript.

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REFERENCES

Akunov, A., Sydykov, A., Toktash, T., Doolotova, A., and Sarybaev, A. (2018). Hemoglobin changes after long-term intermittent work at high altitude. *Front. Physiol.* 9:1552. doi: 10.3389/fphys.2018.01552

Al Riyami, N. B., Banerjee, Y., Al-Waili, K., Rizvi, S. G., Al-Yahyaee, S., Hassan, M. O., et al. (2015). The effect of residing altitude on levels of high-density lipoprotein cholesterol: a pilot study from the Omani Arab Population. *Angiology* 66, 568–573. doi: 10.1177/0003319714544355

Al-Shehri, M. A., Abolfotouh, M. A., Dalak, M. A., and Nwoye, L. D. (2005). Birth anthropometric parameters in high and low altitude areas of Southwest Saudi Arabia. *Saudi Med. J.* 26, 560–565.

Aryal, N., Weatherall, M., Bhatt, Y. K. D., and Mann, S. (2015). Are high altitude populations at high risk of heart disease? A cross-sectional study in Nepal. *Heart Lung Circ.* 24:S109. doi: 10.1016/j.hlc.2015.04.150

Aryal, N., Weatherall, M., Bhatta, Y. K. D., and Mann, S. (2017). Lipid profiles, glycated hemoglobin, and diabetes in people living at high altitude in Nepal. *Int. J. Environ. Res. Public. Health* 14:91041. doi: 10.3390/ijerph14091041

Azad, P., Stobdan, T., Zhou, D., Hartley, I., Akbari, A., Bafna, V., et al. (2017). High-altitude adaptation in humans: from genomics to integrative physiology. *J. Mol. Med.* 95, 1269–1282. doi: 10.1007/s00109-017-1584-7

Beall, C. M. (2006). Andean, Tibetan, and Ethiopian patterns of adaptation to high-altitude hypoxia. *Integr. Comp. Biol.* 46, 18–24. doi: 10.1093/icb/icj004

Beall, C. M. (2007). Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proc. Natl. Acad. Sci. U. S. A.* 104, 8655–8660. doi: 10.1073/pnas.0701985104

Beall, C. M., Brittenham, G. M., Strohl, K. P., Blangero, J., Williams-Blangero, S., Goldstein, M. C., et al. (1998). Hemoglobin concentration of high-altitude Tibetans and Bolivian Aymara. *Am. J. Phys. Anthropol.* 106, 385–400. doi: 10.1002/(SICI)1096-8644(199807)106:3<385::AID-AJPA10>3.0.CO;2-X

Brutsaert, T. (2016). "Why are high altitude natives so strong at high altitude? Nature vs. nurture: genetic factors vs. growth and development," in *Hypoxia*, eds R. C. Roach, P. H. Hackett, and P. D. Wagner (Berlin: Springer), 101–112. doi: 10.1007/978-1-4899-7678-9_7

Brutsaert, T. D., Parra, E. J., Shriver, M. D., Gamboa, A., Rivera-Ch, M., and León-Velarde, F. (2005). Ancestry explains the blunted ventilatory response to sustained hypoxia and lower exercise ventilation of Quechua altitude natives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R225–R234. doi: 10.1152/ajpregu.00105.2005

Burtscher, J., Millet, G. P., and Burtscher, M. (2021). Does living at moderate altitudes in Austria affect mortality rates of various causes? An ecological study. *BMJ Open* 11:e048520. doi: 10.1136/bmjopen-2020-048520

Burtscher, M. (2016). Lower mortality rates in those living at moderate altitude. *Aging* 8, 2603–2604. doi: 10.18632/aging.101057

Cardoso, S., Alfonso-Sánchez, M. A., Valverde, L., Sánchez, D., Zarrabeitia, M. T., Odriozola, A., et al. (2012). Genetic uniqueness of the Waorani tribe from the Ecuadorian Amazon. *Heredity* 108:609. doi: 10.1038/hdy.2011.131

Dhiman, D., Mahajan, S. K., Sharma, S., and Raina, R. (2018). The evolving pattern and outcome of stroke at moderate altitude. *J. Neurosci. Rural Pract.* 9:68. doi: 10.4103/jnrp.jnrp_207_17

Dolma, P., Angchuk, P. T., Jain, V., Dadhwal, V., Kular, D., Williams, D. J., et al. (2021). High-altitude population neonatal and maternal phenotypes associated with birthweight protection. *Pediatr. Res.* 5, 1–6. doi: 10.1038/s41390-021-01593-5

Faeh, D., Gutzwiller, F., and Bopp, M. (2009). Lower mortality from coronary heart disease and stroke at higher altitudes in Switzerland. *Circulation* 120, 495–501. doi: 10.1161/CIRCULATIONAHA.108.819250

Freire, W., Ramírez-Luzuriaga, M., and Belmont, P. (2015). Tomo I: Encuesta Nacional de Salud y Nutrición de la población ecuatoriana de cero a 59 años, ENSANUT-ECU 2012. *Rev. Latinoam. Políticas Acción Pública* 2:117. doi: 10.17141/mundosplurales.1.2015.1914

GAD Oyacachi (2019). *Actualización del plan de desarrollo y Ordenamiento terrritorial de Oyacachi 2014-2019*. Available online at: http://app.sni.gob.ec/sni-link/sni/PORTAL_SNI/data_sigad_plus/sigadplusdocumentofinal/1768098760001_PDyOT%20DIAGNOSTICO%20OYACACHI%201_30-10-2015_23-37-32.pdf (accessed June, 2020).

GAG Limoncocha (2019). *Actualización del plan de desarrollo y ordenamiento territorial de Limoncocha 2014-2019*. Available online at: http://app.sni.gob.ec/sni-link/sni/PORTAL_SNI/data_sigad_plus/sigadplusdocumentofinal/1768086160001_ACTUALIZACION%20PDOT%20LIMONCOCHA%202015%20-%202019_29-10-2015_15-41-36.pdf (accessed June, 2020).

Gonzales, G. F., and Tapia, V. (2013). Association of high altitude-induced hypoxemia to lipid profile and glycemia in men and women living at 4100 m in the Peruvian Central Andes. *Endocrinol. Nutr. Engl. Ed.* 60, 79–86. doi: 10.1016/j.endonu.2012.06.002

Hoke, M. K., and Leatherman, T. L. (2019). Secular trends in growth in the high-altitude district of Nuñoa, Peru 1964–2015. *Am. J. Phys. Anthropol.* 168, 200–208. doi: 10.1002/ajpa.23736

Holmström, P., Mulder, E., Starfelt, V., Lodin-Sundström, A., and Schagatay, E. (2020). Spleen size and function in sherpa living high, sherpa living low and nepalese lowlanders. *Front. Physiol.* 11:647. doi: 10.3389/fphys.2020.00647

Iannotti, L. L., Zavaleta, N., León, Z., and Caulfield, L. E. (2009). Growth and body composition of Peruvian infants in a periurban setting. *Food Nutr. Bull.* 30, 245–253. doi: 10.1177/156482650903000305

Imray, C., Booth, A., Wright, A., and Bradwell, A. (2011). Acute altitude illnesses. *BMJ* 343:d4943. doi: 10.1136/bmj.d4943

INEC (2018). *Anuario de Estadística de Salud: Recursos y Actividades 2017*. Available online at: https://www.ecuadorencifras.gob.ec/documentos/web-inec/Estadisticas_Sociales/Recursos_Actividades_de_Salud/Publicaciones/Anuario_Rec_Act_Salud_2014.pdf (accessed June, 2020).

Julian, C. G., and Moore, L. G. (2019). Human genetic adaptation to high altitude: evidence from the Andes. *Genes* 10:150. doi: 10.3390/genes10020150

Karl, J. P., Cole, R. E., Berryman, C. E., Finlayson, G., Radcliffe, P. N., Kominsky, M. T., et al. (2018). Appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at high altitude, but are not affected by protein intake. *High Alt. Med. Biol.* 19, 156–169. doi: 10.1089/ham.2017.0155

Khalid, M. (1995). Anthropometric comparison between high-and low-altitude Saudi Arabians. *Ann. Hum. Biol.* 22, 459–465. doi: 10.1080/03014469500004122

Khalid, M. E. (2007). The prevalence of abdominal obesity and its associated risk factors in married, non-pregnant women born and living in high altitude, southwestern, Saudi Arabia. *Saudi Med. J.* 28, 1875–1880.

Krüger, H., and Arias-Stella, J. (1970). The placenta and the newborn infant at high altitudes. *Am. J. Obstet. Gynecol.* 106, 586–591. doi: 10.1016/0002-9378(70)90045-1

Leatherman, T. L., Thomas, R. B., Greksa, L. P., and Haas, J. D. (1984). Anthropometric survey of high-altitude Bolivian porters. *Ann. Hum. Biol.* 11, 253–256. doi: 10.1080/03014468400007131

Li, L., and Zhao, X. (2015). Comparative analyses of fecal microbiota in Tibetan and Chinese Han living at low or high altitude by barcoded 454 pyrosequencing. *Sci. Rep.* 5, 1–10. doi: 10.1038/srep14682

Lichty, J. A., Ting, R. Y., Bruns, P. D., and Dyar, E. (1957). Studies of babies born at high altitude: I. Relation of altitude to birth weight. *AMA J. Dis. Child.* 93, 666–678. doi: 10.1001/archpedi.1957.02060040668009

Lin, B. Y., Genden, K., Shen, W., Wu, P.-S., Yang, W.-C., Hung, H.-F., et al. (2018). The prevalence of obesity and metabolic syndrome in Tibetan immigrants living in high altitude areas in Ladakh, India. *Obes. Res. Clin. Pract.* 12, 365–371. doi: 10.1016/j.orcp.2017.03.002

Lundby, C., Sander, M., Van Hall, G., Saltin, B., and Calbet, J. A. (2006). Maximal exercise and muscle oxygen extraction in acclimatizing lowlanders and high altitude natives. *J. Physiol.* 573, 535–547. doi: 10.1113/jphysiol.2006.106765

Merrill, R. M. (2020). Explaining the inverse association between altitude and obesity. *J. Obes.* 2020:1946723. doi: 10.1155/2020/1946723

Miggitsch, E. M., Trapp, M., Rohrer, P. M., Velik, R., Domej, W., Pieringer, W., et al. (2009). Bradycardia at high altitude. *Alpinmedizinischer Rundbr.* 40, 38–45.

Milledge, J. (2020). Hypobaria: high altitude, aviation physiology, and medicine. *Cotes' Lung Funct.* 2020, 615–637. doi: 10.1002/9781118597309.ch32

Miranda, J. J., Bernabe-Ortiz, A., Gilman, R. H., Smeeth, L., Malaga, G., Wise, R. A., et al. (2019). Multimorbidity at sea level and high-altitude urban and rural settings: the CRONICAS Cohort Study. *J. Comorbidity* 9:2235042X19875297. doi: 10.1177/2235042X19875297

Mohanna, S., Baracco, R., and Seclén, S. (2006). Lipid profile, waist circumference, and body mass index in a high altitude population. *High Alt. Med. Biol.* 7, 245–255. doi: 10.1089/ham.2006.7.245

Moore, L. G. (2001). Human genetic adaptation to high altitude. *High Alt. Med. Biol.* 2, 257–279. doi: 10.1089/152702901750265341

Moore, L. G. (2017a). Human genetic adaptation to high altitudes: current status and future prospects. *Quat. Int. J. Int. Union Quat. Res.* 461, 4–13. doi: 10.1016/j.quaint.2016.09.045

Moore, L. G. (2017b). Measuring high-altitude adaptation. *J. Appl. Physiol.* 123, 1371–1385. doi: 10.1152/japplphysiol.00321.2017

Moore, L. G., Charles, S. M., and Julian, C. G. (2011). Humans at high altitude: hypoxia and fetal growth. *Respir. Physiol. Neurobiol.* 178, 181–190. doi: 10.1016/j.resp.2011.04.017

Moore, L. G., Niermeyer, S., and Zamudio, S. (1998). Human adaptation to high altitude: regional and life-cycle perspectives. *Am. J. Phys. Anthropol.* 27, 25–64. doi: 10.1002/(SICI)1096-8644(1998)107:27<25::AID-AJPA3>3.0.CO;2-L

Ortiz-Prado, E., Dunn, J. F., Vasconez, J., Castillo, D., and Viscor, G. (2019). Partial pressure of oxygen in the human body: a general review. *Am. J. Blood Res.* 9:1–14.

Ortiz-Prado, E., Simbaña, K., Gómez, L., Henriquez-Trujillo, A. R., Cornejo-Leon, F., Vasconez, E., et al. (2017). The disease burden of suicide in Ecuador, a 15 years' geodemographic cross-sectional study (2001–2015). *BMC Psychiatry* 17:342. doi: 10.1186/s12888-017-1502-0

Pasha, M. Q., and Newman, J. H. (2010). High-altitude disorders: pulmonary hypertension: pulmonary vascular disease: the global perspective. *Chest* 137, 13S-19S. doi: 10.1378/chest.09-2445

Pennisi, E. (2018). High altitude may have driven short stature in Peruvians. *Science* 360:696. doi: 10.1126/science.360.6390.696

Pereira, R., Phillips, C., Pinto, N., Santos, C., Santos, S. E. B., dos Amorim, A., et al. (2012). Straightforward inference of ancestry and admixture proportions through ancestry-informative insertion deletion multiplexing. *PLoS ONE* 7:e29684. doi: 10.1371/journal.pone.0029684

Pritchard, J. K., Stephens, M., and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959. doi: 10.1093/genetics/155.2.945

Ranhotra, H. S., and Sharma, R. (2010). Moderately high altitude habitation modulates lipid profile and alkaline phosphatase activity in aged Khasis of Meghalaya. *Indian J. Clin. Biochem.* 25, 51–56. doi: 10.1007/s12291-010-0011-4

Rausch, L. K., Hofer, M., Pramsohler, S., Kaser, S., Ebenbichler, C., Haacke, S., et al. (2018). Adiponectin, leptin and visfatin in hypoxia and its effect for weight loss in obesity. *Front. Endocrinol.* 9:615. doi: 10.3389/fendo.2018.00615

Samaja, M., Crespi, T., Guazzi, M., and Vandegriff, K. D. (2003). Oxygen transport in blood at high altitude: role of the hemoglobin–oxygen affinity and impact of the phenomena related to hemoglobin allosterism and red cell function. *Eur. J. Appl. Physiol.* 90, 351–359. doi: 10.1007/s00421-003-0954-8

Santangelo, R., González-Andrade, F., Børsting, C., Torroni, A., Pereira, V., and Morling, N. (2017). Analysis of ancestry informative markers in three main ethnic groups from Ecuador supports a trihybrid origin of Ecuadorians. *Forensic Sci. Int. Genet.* 31, 29–33. doi: 10.1016/j.fsigen.2017.08.012

Sherpa, L. Y., Deji, Stigum, H., Chongsuvivatwong, V., Luobu, O., Thelle, D. S., et al. (2011). Lipid profile and its association with risk factors for coronary heart disease in the highlanders of Lhasa, Tibet. *High Alt. Med. Biol.* 12, 57–63. doi: 10.1089/ham.2010.1050

Siqués, P., Brito, J., León-Velarde, F., Barrios, L., De La Cruz, J. J., López, V., et al. (2007b). Hematological and lipid profile changes in sea-level natives after exposure to 3550-m altitude for 8 months. *High Alt. Med. Biol.* 8, 286–295. doi: 10.1089/ham.2007.8405

Smith, L. (2005). New AHA recommendations for blood pressure measurement. *Am. Fam. Physician* 72, 1391–1398. Available online at: https://www.aafp.org/afp/2005/1001/p1391.html

Stobdan, T., Akbari, A., Azad, P., Zhou, D., Poulsen, O., Appenzeller, O., et al. (2017). New insights into the genetic basis of Monge's disease and adaptation to high-altitude. *Mol. Biol. Evol.* 34, 3154–3168. doi: 10.1093/molbev/msx239

Storz, J. F. (2007). Hemoglobin function and physiological adaptation to hypoxia in high-altitude mammals. *J. Mammal.* 88, 24–31. doi: 10.1644/06-MAMM-S-199R1.1

Toscanini, U., Gaviria, A., Pardo-Seco, J., Gómez-Carballa, A., Moscoso, F., Vela, M., et al. (2018). The geographic mosaic of Ecuadorian Y-chromosome ancestry. *Forensic Sci. Int. Genet.* 33, 59–65. doi: 10.1016/j.fsigen.2017.11.011

Toselli, S., Tarazona-Santos, E., and Pettener, D. (2001). Body size, composition, and blood pressure of high-altitude Quechua from the Peruvian Central Andes (Huancavelica, 3,680 m). *Am. J. Hum. Biol.* 13, 539–547. doi: 10.1002/ajhb.1086

Tyagi, R., Tungdim, M. G., Bhardwaj, S., and Kapoor, S. (2008). Age, altitude and gender differences in body dimensions. *Anthropol. Anz.* 66, 419–434. doi: 10.1127/aa/66/2008/419

Vats, P., Ray, K., Majumadar, D., Amitabh, Joseph, D. A., Bayen, S., et al. (2013). Changes in cardiovascular functions, lipid profile, and body composition at high altitude in two different ethnic groups. *High Alt. Med. Biol.* 14, 45–52. doi: 10.1089/ham.2012.1071

Walsh, P. S., Metzger, D. A., and Higuchi, R. (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10, 506–513.

West, J. B. (2006). Human responses to extreme altitudes. *Integr. Comp. Biol.* 46, 25–34. doi: 10.1093/icb/icj005

West, J. B. (2012). High-altitude medicine. *Am. J. Respir. Crit. Care Med.* 186, 1229–1237. doi: 10.1164/rccm.201207-1323CI

West, J. B., Schoene, R. B., Milledge, J. S., and Ward, M. P. (2007). *High Altitude Medicine and Physiology*. Hodder Arnold London. Available online at: http://www.jrnms.com/wp-content/uploads/2014/05/JRNMS-95-1-40-43.pdf (accessed August 16, 2016).

Westerterp, K. R. (2001). Energy and water balance at high altitude. *Physiology* 16, 134–137. doi: 10.1152/physiologyonline.2001.16.3.134

Winslow, R. M. (1984). "High-altitude polycythemia," in *High Altitude and Man* ed J. B. West (Berlin: Springer), 163–172. doi: 10.1007/978-1-4614-7525-5_15

Zambrano, A. K., Gaviria, A., Cobos-Navarrete, S., Gruezo, C., Rodríguez-Pollit, C., Armendáriz-Castillo, I., et al. (2019). The three-hybrid genetic composition of an Ecuadorian population using AIMs-InDels compared with autosomes, mitochondrial DNA and Y chromosome data. *Sci. Rep.* 9, 1–8. doi: 10.1038/s41598-019-45723-w

Zamudio, S. (2003). The placenta at high altitude. *High Alt. Med. Biol.* 4, 171–191. doi: 10.1089/152702903322022785

Zangari, M., Fink, L., Tolomelli, G., Lee, J. C. H., Stein, B. L., Hickman, K., et al. (2013). Could hypoxia increase the prevalence of thrombotic complications in polycythemia vera? *Blood Coagul. Fibrinolysis* 24, 311–316. doi: 10.1097/MBC.0b013e32835bfdb9

Zhong, R., Liu, H., Wang, H., Li, X., He, Z., Gangla, M., et al. (2015). Adaption to high altitude: an evaluation of the storage quality of suspended red blood cells prepared from the whole blood of Tibetan plateau migrants. *PLoS ONE* 10:e0144201. doi: 10.1371/journal.pone.0144201

Zhuang, J., Droma, T., Sun, S., Janes, C., McCullough, R. E., McCullough, R. G., et al. (1993). Hypoxic ventilatory responsiveness in Tibetan compared with Han residents of 3,658 m. *J. Appl. Physiol.* 74, 303–311. doi: 10.1152/jappl.1993.74.1.303

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