Free Radical Biology and Medicine 223 (2024) 193–198

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

# Hyperoxic recovery interferes with the metabolic imprint of hypoxic exercise

Johannes Burtscher [a,*], Giuseppe Paglia [b], Vanna Denti [b], Martin Faulhaber [c], Günter Weiss [d], Wolfgang Schobersberger [e,f], Tobias Dünnwald [e,**]

[a] Institute of Sport Sciences, University of Lausanne, Lausanne, Switzerland
[b] School of Medicine and Surgery, University of Milano-Bicocca, Vedano al Lambro (MB), Italy
[c] Department of Sport Science, University of Innsbruck, Innsbruck, Austria
[d] Department of Internal Medicine II, Medical University of Innsbruck, Innsbruck, Austria
[e] Institute for Sports Medicine, Alpine Medicine and Health Tourism (ISAG), UMIT TIROL - Private University for Health Sciences and Health Technology, Hall in Tirol, Austria
[f] Tirol-Kliniken GmbH, Innsbruck, Austria

ARTICLE INFO

*Keywords:*
Hyperoxia
Hypoxia
Metabolism
Acclimatization
exercise
High altitude illnesses

ABSTRACT

Supplemental oxygen (hyperoxia) improves physical performance during hypoxic exercise. Based on the analysis of metabolome and iron homeostasis from human athlete blood samples, we show that hyperoxia during recovery periods interferes with metabolic alterations following hypoxic exercise. This may impair beneficial adaptations to exercise and/or hypoxia and highlights risks of oxygen supplementation in hypoxia.

## 1. Introduction

Reduced ambient oxygen availability (hypoxia), e.g., at high altitude, induces cellular and systemic responses to maintain function and performance. Nevertheless, acute hypoxia reduces exercise capacity and poses health threats [1]. Oxygen supplementation during physical exercise – and possibly before hypoxia exposure [2] – may reduce these risks of exercising at extreme altitude. The use of supplemental oxygen thus is a strategy to enhance performance at high altitude [3], but whether it is efficient if applied during recovery is elusive. We recently reported that skiing-relevant high-intensity leg exercise in hypoxia induces long-term alterations of the blood metabolome [4] and that hyperoxic periods during recovery between sets did not improve exercise performance [5]. Here we present blood metabolomic data suggesting that hyperoxic recovery blunts metabolic adaptations to hypoxic exercise. Our results highlight risks of the increasing commercialization and insufficiently investigated athletic utilization of oxygen supplementation.

## 2. Materials and methods

### 2.1. Subjects

Eleven male trained athletes (mean (SD) age: 30.4 (6.0) years, body height: 181(5.0) cm, weight: 76.4 (8.7) kg, $VO_2$max: 53.9 (5.9) ml $min^{-1}$, $HR_{max}$ 190 (8) bpm) volunteered to participate in this randomized, single blinded cross-over study. All athletes completed an extensive medical examination including a graded symptom-limited bicycle ergometer test (Lode B.V., Groningen, Netherlands) with gas analyses (Care Fusion, Vyntus CPX, Hoechberg, Germany). The Ethical Committee of the Medical University of Innsbruck (Austria) approved the study. Subjects gave their written informed consent after having obtained verbal and written information about the aims, procedures and risks of the study. The study was carried out in line with the ethical standards laid down in the 1975 of the Declaration of Helsinki.

### 2.2. Experimental design

Subjects were exposed to normobaric hypoxia (Low Oxygen

* Corresponding author.
** Corresponding author.
*E-mail addresses:* johannes.burtscher@unil.ch (J. Burtscher), tobias.duennwald@umit-tirol.at (T. Dünnwald).

https://doi.org/10.1016/j.freeradbiomed.2024.07.024
Received 16 May 2024; Received in revised form 11 July 2024; Accepted 23 July 2024
Available online 26 July 2024

**Abbreviations;**

| | |
|---|---|
| AMS | acute mountain sickness |
| EDTA | Ethylenediaminetetraacetic acid |
| ER | enrichment ratio |
| ETS | electron transfer system |
| HS | hyperoxic recovery settings |
| LC/MS | Liquid chromatography–mass spectrometry |
| NAD+ | oxidized nicotinamide adenine dinucleotide; |
| NAD(H) | nicotinamide adenine dinucleotide (oxidized and reduced) |
| NHS | non-hyperoxic recovery settings |
| VO2max | maximal oxygen uptake |
| HRmax | maximal heart rate |
| TCA | tricarboxylic acid cycle |
| QC | quality control |

Chamber, Berlin, Germany) on 2 different days, interspersed by a 2-week wash-out period. Following a 60 min-adaptation phase (including a 10 min warm-up) at simulated altitude of 3500 m, an exercise intervention consisting of 5 sets of flywheel exercise (90 s each), separated by 4 recovery periods of 15 min was carried out. Each set was composed of repeated high-intensity leg exercises (i.e., concentric-eccentric bilateral side-squats at maximum concentric velocity) on a flywheel device (k-Box4 Pro, Exxentric AB, Sweden) (for details see Ref. [5]). The same exercise protocol was performed on both days, however, using 2 different recovery strategies: during the recovery period the subjects either breathed 100 % oxygen (hyperoxic recovery setting - HS) or hypoxic air (fraction of inspired oxygen $F_IO_2 = 0.146$, non-hyperoxic recovery setting - NHS) on day 1 and day 2, respectively. Subjects were randomly allocated to 1 of the 2 conditions (i.e., HS or NHS) on the first day. For each flywheel set, performance (power output) was determined. Blood samples were collected before (pre, 0), directly after (post 1) and 7 days (post 2) after the exercise intervention (see Fig. 1A).

### 2.3. Blood analyses and markers of iron homeostasis

Venous blood sampling was performed on the antecubital vein in the forearm (1 × 5 mL EDTA sample) before and at 2 timepoints after the exercise intervention on both days, at the same time of the day. Thereafter, blood was inverted 5 times before blood EDTA samples were centrifuged at 2.500 rpm for 10 min at room temperature. Blood plasma was then stored at −80 °C until being analyzed.

Fully automated clinical standard assessments of iron homeostasis markers were performed by a routine laboratory.

### 2.4. Untargeted metabolomics analysis

The plasma polar metabolome was analyzed using as previously described [4]. In brief, plasma samples were analyzed using an LC/MS 6546 platform (Agilent Technologies, Palo Alto, CA, USA). Polar metabolites were resolved by hydrophilic interaction liquid chromatography (HILIC) using an Acquity amide column, 100 × 2.1 mm, 1.7 μm (Waters, Milford, MA, USA). All samples were analyzed 3 times: once in positive ionization mode and twice in negative ionization mode using acidic and basic chromatographic conditions.

Protein precipitation was achieved by adding cold acetonitrile (150 μL) to 50 μL of plasma and centrifuged at 15000 g and 2 μL of supernatant were injected in the LC-MS system.

Pooled quality control (QC) samples were obtained by pooling together 10 μL from each sample and were injected every 15 samples during the analysis to evaluate the performance of the LC-MS system. Finally, 4 injections of pooled QC samples were performed at the end of the analysis in Data-dependent acquisition (DDA) mode to acquire fragmentation spectra.

Data acquisition and analysis was done using the Agilent MassHunter software and Mass Profiler Professional. Annotated features were also integrated using Agilent MassHunter Quantitative software. 115 polar metabolites were annotated based on accurate mass, MS/MS, isotopic pattern and retention times against our in-house database and/or online databases, including HMDB (Wishart et al., 2018) and METLIN (Guijas et al., 2018). Metabolites with a relative standard deviation (RSD%) higher than 30 in pooled QC samples were removed and not considered for further analysis.

### 2.5. Statistics

Univariate and multivariate statistical analysis was perfomed using MetaboAnalyst (https://doi.org/10.1038/s41596-022-00710-w). Data was first normalized by the sum of the features, then was log transformed and scaled by unit variance scaling method before applying univariate and multivariate analysis. Volcano Plots were obtained using a fold change threshold of 2 and a p value threshold of 0.05 after false discovery rate.

For power output across sessions (Fig. 1B), simple linear regressions were calculated, and significant slopes were evaluated. Two-way repeated measures ANOVAs (with Geisser-Greenhouse correction) were used to compare measures of iron homeostasis (Fig. 2J–Q), in case of significant interaction effects, Tukey's post-hoc tests were calculated. Regressions and ANOVAs were performed with GraphPad Prism version 10.2.1 for Windows, GraphPad Software, Boston, Massachusetts USA.

## 3. Results and discussion

The exercise intervention was performed on 2 different examination days in normobaric hypoxia (simulating an altitude of 3500 m) [5] (Fig. 1A). During the recovery period the subjects either breathed hyperoxic (HS) or hypoxic air (NHS). While the power output between the groups was similar across all 5 sets of exercise [5], the analysis of the change in power output (linear regression) from set 1 to 5 revealed a significant progressive improvement in NHS (slope = 4.6, p = 0.03), which was not observed in HS (slope = 1.4, p = 0.55) (Fig. 1B). This suggests that a hypoxic recovery is required for appropriate physiological responses to limit performance-reduction in hypoxia. We studied the blood metabolome at 3 time points (before, directly after and 1 week after the intervention, Fig. 1C) to assess, whether hyperoxic recovery affects acute and long-lasting adaptive responses to hypoxic exercise [1, 6]. The metabolic changes directly after the exercise intervention are depicted in the volcano plots in Fig. 1D. The most notable short-term effects (directly after the training session) include changes in metabolites associated with energy and lipid metabolism (Fig. 2A and B). Pathways related to the mitochondrial electron transfer system (ETS), tricarboxylic acid cycle (TCA), oxidation of branched-chained fatty acids, increased transfer of acetyl groups in mitochondria (suggesting enhanced acetyl-CoA supply for the TCA) and of increased carnitine synthesis, contributing to improved fatty acid transport into mitochondria, changed more in the HS condition. Together, these results likely reflect maintained oxidative energy generation by beta oxidation, maintenance of TCA cycle efficiency and oxidative phosphorylation due to oxygen supplementation. Pathway activation linked to glycolysis and the Warburg effect was also higher in HS than in NHS. Those changes suggest better cellular energy provision in HS directly after the exercise intervention, which, however, did not translate into functional benefits during exercise (Fig. 1B and [5]).

Conversely, more pronounced changes were observed in pathways related to gluconeogenesis and Cahill cycle and small differences in enrichment for lipid metabolism in NHS. These changes reflect increased glucose utilization, an expected metabolic response in hypoxia

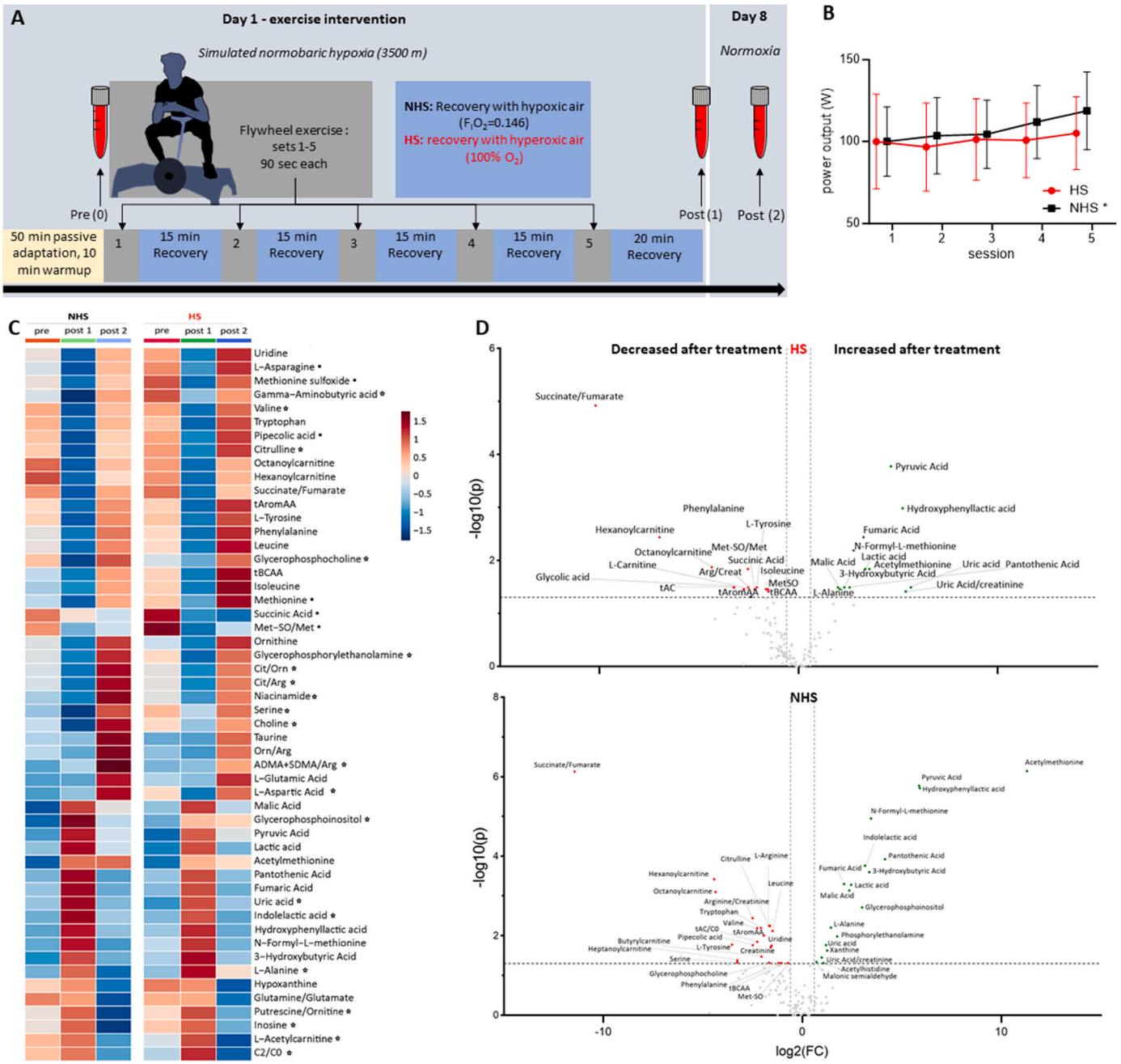


**Fig. 1. Exercise protocol and short-term effects.** The protocol of the exercise intervention (A) and the evolution of mean power output (normalized to set 1) during the intervention: hypoxic recovery setting (NHS) and hyperoxic recovery setting (HS) are compared (B). (C) Metabolomic analysis from blood samples as collected in (A). (D) Volcano plots assessing short-term effects. * denotes a significant non-zero slope ($p < 0.03$), significant differences only in HS are shown by rectangles and only in NHS by stars in (C).

indicating sustained metabolic reliance on glucose in hypoxia [1,7] and potentially a protective molecular response to hypoxia [8].

Considering the lack of functional improvement during the training sessions in NHS, we propose that oxygen supplementation in the recovery phase of hypoxic exercise rather impairs metabolic responses that are required for successful adaptations to exercise and/or hypoxia. Blood metabolome and iron homeostasis changed differently in HS and NHS 1 week after the hypoxic exercise intervention, indicating modulation of adaptive processes in response to hypoxic exercise by oxygen supplementation. Arginine/creatinine, L-alanine and other glucogenic amino acid-levels were increased in HS, with opposing effects in NHS (Fig. 1C). Principal component analysis on the blood metabolome revealed a clear pattern of metabolic alterations 7 days after the intervention in NHS but not in HS (Fig. 3A and B). Quantitative enrichment analyses showed that the main differences between HS and NHS were related to nicotinamide adenine dinucleotide (NAD(H)) metabolism-related pathways (Fig. 3C). The variation of exemplary metabolites across all time points, normalized to the median of the measurements before the intervention is shown in Supplementary Fig. 1 (statistics in Fig. 1C/volcano plots). Circulating lactic acid (SFig. 1A) and beta-hydroxybutyrate (SFig. 1B) were transiently increased in both conditions after the training intervention (indicating increased glycolysis and ketosis). The phospholipid metabolism-related glycerophosphoinositol (SFig. 1C) was specifically upregulated in NHS directly after the exercise intervention (similar trend for phosphorylethanolamine (SFig. 1D)). Increased choline SFig. 1E) and niacinamide

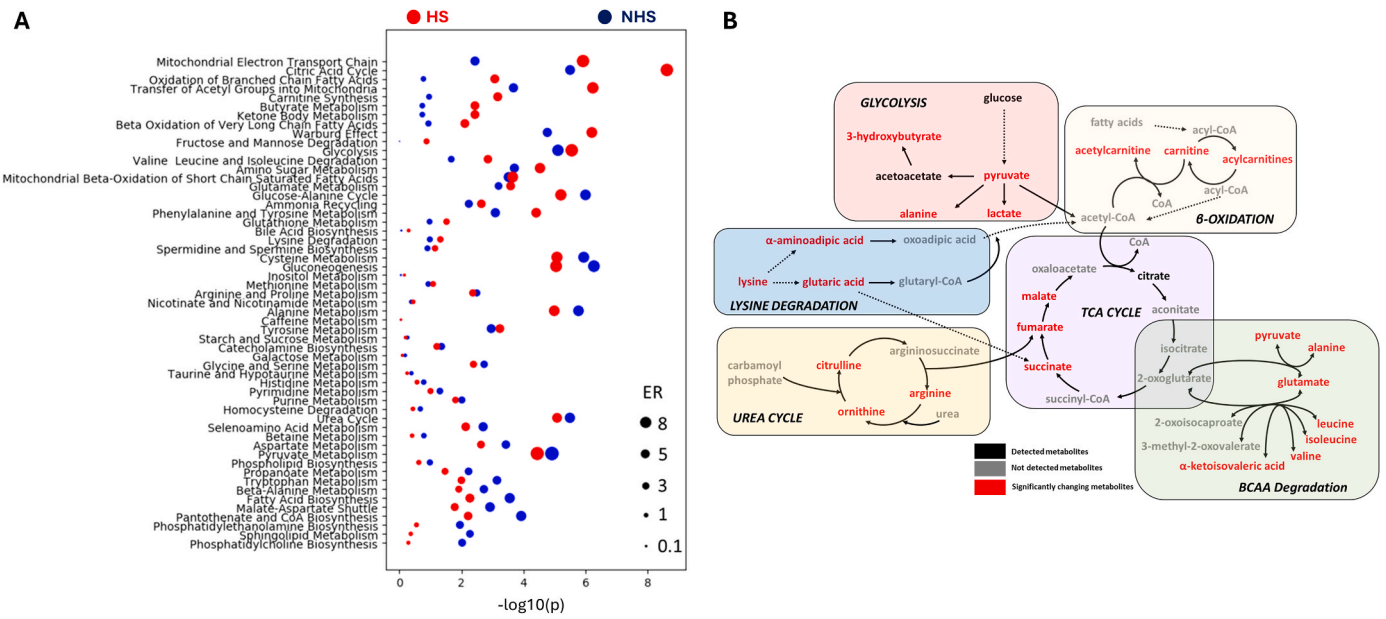


**Fig. 2. Enrichment analysis and metabolic pathways directly after the exercise intervention.** (A) Quantitative enrichment analysis: ER (enrichment ratio) and (B) metabolic pathways were sorted by ΔER (ER difference between hyperoxic recovery setting (HS) before and directly after the exercise intervention (post1) compared against the hypoxic recovery setting (NHS) before and directly after the exercise intervention).

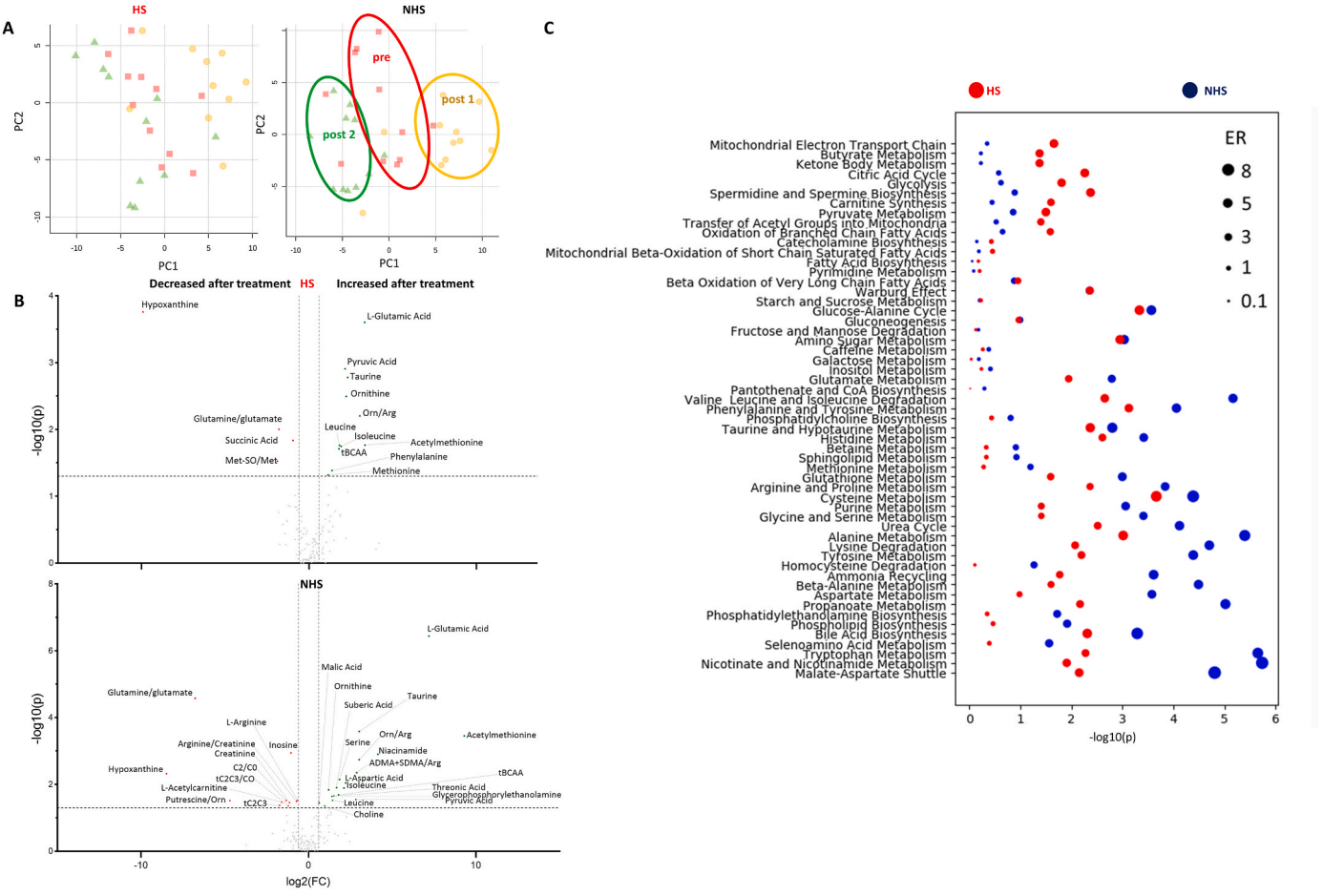


**Fig. 3. Long-term effects of oxygen supplementation on blood metabolome and iron homeostasis.** (A) Principal component (PC) analysis shows clearer patterns in metabolite regulation 7 days after the intervention (post 2) in the hypoxic recovery group (NHS). PC1s are 21 and 20.9 % and PC2s are 15.4 and 12.9 % for the hyperoxic recovery group (HS) and NHS, respectively. (B) Volcano plots comparing metabolite levels from before the exercise and 1 week after. (C) Quantitative enrichment analyses for HS and NHS at post 2. ER (enrichment ratio) and metabolic pathways were sorted by ΔER (see Fig. 1 and methods).

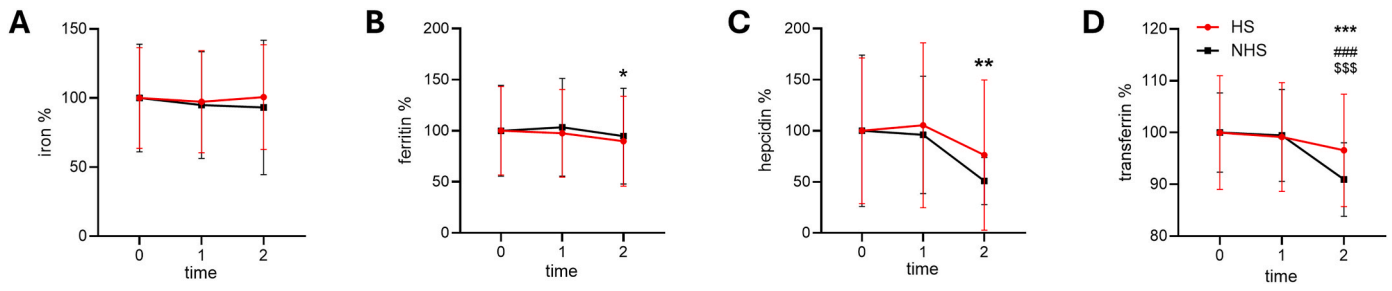


**Fig. 4. Iron-homeostasis-related measurements**. (A)–(D) are normalizations to pre-exercise values (for absolute values see Supplementary Fig. 2). Two-way repeated measurement ANOVAs were performed. * denotes significant main time effects, # significant Tukey's post-hoc tests vs pre (time 1) and $ vs post 1 (time 2).

(SFig. 1F) levels in NHS 1 week after hypoxic exercise may indicate protection from future hypoxia. Choline protects vessels from hypoxic insults [9] and higher levels of niacinamide suggest improved $NAD^+$ availability, increasing resilience to future hypoxic or metabolic stress [10].

Given the role of iron homeostasis in hypoxia adaptations [11], we compared iron-related parameters in HS and NHS blood (normalized in Fig. 4, absolute changes in SFig. 2. While overall iron levels were not changed in either condition (Fig. 4A), a drop of the iron-storing protein ferritin (Fig. 4B) and the iron-uptake regulator hepcidin (Fig. 4C) were observed 1 week after the exercise intervention to similar extent in HS and NHS (2-way repeated measurements ANOVAs, main effects of time $p = 0.033$ and $p = 0.002$, respectively). These effects are likely due to increased hypoxia-induced erythropoiesis (requiring iron) that leads to the downregulation of hepcidin [12]. Transferrin-levels (Fig. 4D) were reduced in NHS compared to HS (2way repeated measurements ANOVAs, interaction effect $p = 0.008$), 1 week after the exercise intervention. Since the reduction of transferrin may protect from hypoxia-induced thromboembolic events [13], this could be another adaptive mechanism reducing detrimental effects of hypoxic exposure.

In summary, blood metabolomes determined directly after the exercise intervention differed primarily by upregulated pathways related to ETS, fatty acid oxidation, TCA and glycolysis, in HS compared to NHS. In contrast, lipid metabolism was slightly upregulated in NHS. Contrary to our initial hypothesis, exercise capacity did not improve in HS [5]: instead, performance increased more steeply across the 5 sets of exercise in NHS. Seven days after the exercise intervention, the metabolic profile was more clearly distinguishable between NHS and HS with major metabolic differences related to the major redox regulator couple NAD(H) [14]. These results indicate that hypoxic training induced robust metabolic adaptation that may have been attenuated by oxygen supplementation during recovery. Such metabolic adaptation could be crucial for long-term exercise benefits and/or metabolic adaptation to hypoxia, including acclimatization that protects from high-altitude illnesses, such as acute mountain sickness (AMS). Our results thus indicate that hyperoxic recovery may attenuate the protection from acclimatization to altitude, increasing the risk for hypoxic injury. Considering the growing number of high-altitude sojourners, this is a major public health concern [15].

Upregulation of hypoxanthine and acetylcarnitine, may predict AMS [16] and both were decreased in NHS but only acetylcarnitine in HS (Fig. 1C), suggesting better acclimatization and protection from AMS without hyperoxic recovery.

The notion of impaired hypoxia adaptation by hyperoxia is also in line with an hyperoxia-induced inhibition of carotid body chemoreceptors [17], the main mediators of autonomic systemic hypoxia responses [7]. Since potential negative effects of oxygen supplementation in hypoxic exercise are currently insufficiently evaluated, our results call for in-depth investigation of potential associated safety and diminished efficiency consequences.

Due to the serendipitous nature of the presented findings, important open questions remain. Redox regulation is importantly involved in adaptation to hypoxia and hyperoxia [1] as well as to exercise [18] and the differences in NAD(H) metabolism between hypoxic and hyperoxic recovery observed in the present study indicate an importance of redox regulation. Moreover, we detected significantly increased levels of methionine sulfoxide only in HS (Fig. 1C), a molecule resulting from oxidation of methionine by reactive oxygen species (ROS) and/or reactive nitrogen species [19]. This suggests increased production of ROS in hyperoxic recovery conditions. The potential mechanistic roles of NAD(H) metabolism, ROS and oxidative injury in the interference with hypoxic adaptation are promising avenues for more focused studies in the future.

## Funding

This study was funded by the "Tiroler Wissenschaftsförderung - TWF" (project no. F.16520/5–2019).

## Data availability

All metabolomic data are publicly accessible on the MassIVE platform: https://doi.org/10.25345/C50V89T90. Ethical reviews will be provided upon request.

## CRediT authorship contribution statement

**Johannes Burtscher:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Giuseppe Paglia:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vanna Denti:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Martin Faulhaber:** Writing – review & editing, Conceptualization. **Günter Weiss:** Writing – review & editing, Investigation. **Wolfgang Schobersberger:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Tobias Dünnwald:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare no competing interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2024.07.024.

## References


[1] J. Burtscher, R.T. Mallet, V. Pialoux, G.P. Millet, M. Burtscher, Adaptive responses to hypoxia and/or hyperoxia in humans, Antioxidants Redox Signal. (2022) 887–912.

[2] J. You, X. Chen, M. Zhou, H. Ma, Q. Liu, C. Huang, Hyperbaric oxygen preconditioning for prevention of acute high-altitude diseases: fact or fiction? Front. Physiol. 14 (2023) 1019103.

[3] M. Faulhaber, S. Schneider, L.K. Rausch, T. Dünnwald, V. Menz, H. Gatterer, M. D. Kennedy, W. Schobersberger, Repeated short-term bouts of hyperoxia improve aerobic performance in acute hypoxia, J Strength Cond Res 37 (10) (2023) 2016–2022.

[4] T. Dünnwald, G. Paglia, G. Weiss, V. Denti, M. Faulhaber, W. Schobersberger, H. Wackerhage, High intensity concentric-eccentric exercise under hypoxia changes the blood metabolome of trained athletes, Front. Physiol. 13 (2022) 904618.

[5] T. Dünnwald, D. Morawetz, M. Faulhaber, H. Gatterer, C. Birklbauer, A. Koller, G. Weiss, W. Schobersberger, Supplemental O 2 during recovery does not improve repeated maximal concentric-eccentric strength-endurance performance in hypoxia, J Strength Cond Res 36 (11) (2022) 3065–3073.

[6] J. Burtscher, T. Citherlet, A. Camacho-Cardenosa, M. Camacho-Cardenosa, A. Raberin, B. Krumm, E. Hohenauer, M. Egg, M. Lichtblau, J. Müller, E. A. Rybnikova, H. Gatterer, T. Debevec, S. Baillieul, G. Manferdelli, T. Behrendt, L. Schega, H. Ehrenreich, G.P. Millet, M. Gassmann, C. Schwarzer, O. Glazachev, O. Girard, S. Lalande, M. Hamlin, M. Samaja, K. Hüfner, M. Burtscher, G. Panza, R. T. Mallet, Mechanisms underlying the health benefits of intermittent hypoxia conditioning, J Physiol (2023) online ahead of print.

[7] R.T. Mallet, J. Burtscher, V. Pialoux, Q. Pasha, Y. Ahmad, G.P. Millet, M. Burtscher, Molecular mechanisms of high-altitude acclimatization, Int. J. Mol. Sci. 24 (2) (2023) 1698.

[8] J. Burtscher, G. Pepe, N. Maharjan, N. Riguet, A. Di Pardo, V. Maglione, G.P. Millet, Sphingolipids and impaired hypoxic stress responses in Huntington disease, Prog. Lipid Res. 90 (2023) 101224.

[9] L.C. Zhang, X. Jin, Z. Huang, Z.N. Yan, P.B. Li, R.F. Duan, H. Feng, J.H. Jiang, H. Peng, W. Liu, Protective effects of choline against hypoxia-induced injuries of vessels and endothelial cells, Exp. Ther. Med. 13 (5) (2017) 2316–2324.

[10] P.K. Shetty, F. Galeffi, D.A. Turner, Nicotinamide pre-treatment ameliorates NAD (H) hyperoxidation and improves neuronal function after severe hypoxia, Neurobiol. Dis. 62 (2014) 469–478.

[11] M. Gassmann, M.U. Muckenthaler, Adaptation of iron requirement to hypoxic conditions at high altitude, Journal of applied physiology (Bethesda, Md. 119 (12) (2015) 1432–1440, 1985.

[12] Q. Liu, O. Davidoff, K. Niss, V.H. Haase, Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis, J. Clin. Invest. 122 (12) (2012) 4635–4644.

[13] M. Li, X. Tang, Z. Liao, C. Shen, R. Cheng, M. Fang, G. Wang, Y. Li, S. Tang, L. Xie, Z. Zhang, P.M. Kamau, J. Mwangi, Q. Lu, Y. Li, Y. Wang, D.T. MacKeigan, E. G. Cerenzia, H. Ni, R. Lai, Hypoxia and low temperature upregulate transferrin to induce hypercoagulability at high altitude, Blood 140 (19) (2022) 2063–2075.

[14] W. Xiao, R.S. Wang, D.E. Handy, J. Loscalzo, NAD(H) and NADP(H) redox couples and cellular energy metabolism, Antioxid Redox Signal 28 (3) (2018) 251–272.

[15] H. Gatterer, F.C. Villafuerte, S. Ulrich, S.S. Bhandari, L.E. Keyes, M. Burtscher, Altitude illnesses, Nat Rev Dis Primers 10 (1) (2024) 43.

[16] I. Sibomana, D.P. Foose, M.L. Raymer, N.V. Reo, J.P. Karl, C.E. Berryman, A. J. Young, S.M. Pasiakos, C.A. Mauzy, Urinary metabolites as predictors of acute mountain sickness severity, Front. Physiol. 12 (2021) 709804.

[17] M. Sinski, J. Lewandowski, J. Przybylski, P. Zalewski, B. Symonides, P. Abramczyk, Z. Gaciong, Deactivation of carotid body chemoreceptors by hyperoxia decreases blood pressure in hypertensive patients, Hypertens. Res. 37 (9) (2014) 858–862.

[18] M. Ristow, K. Zarse, A. Oberbach, N. Klöting, M. Birringer, M. Kiehntopf, M. Stumvoll, C.R. Kahn, M. Blüher, Antioxidants prevent health-promoting effects of physical exercise in humans, Proc Natl Acad Sci U S A 106 (21) (2009) 8665–8670.

[19] B.C. Lee, V.N. Gladyshev, The biological significance of methionine sulfoxide stereochemistry, Free Radic. Biol. Med. 50 (2) (2011) 221–227.