Mechanisms of hypoxia (in)tolerance in prematurely born adults

Manferdelli Giorgio

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Institut des Sciences du Sport

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Master of Research at the University of the West of Scotland

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Prof. Christopher Newman

"The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day."

Albert Einstein

Acknowledgements

A PhD is a unique time in life. It represents a unique opportunity to learn, grow, and develop your entrepreneurial spirit, but it is also a long process with several up and downs. In short, you imagine projects, torture your supervisor trying to convince him and obtain his consent, realize research protocols, collect experimental data, analyze uncountable data set, discuss your results, and finally publish your work. All these tasks are supported by a multitude of people giving their time to share their knowledge and provide invaluable help and support.

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List of publications

Publication list is organized in three sections: a) publications that are part of this thesis; b) publications within the research project; and c) publications outside the research project.

Publications that are part of the thesis

1. Manferdelli G, Narang BJ, Pialoux V, Giardini G, Debevec T & Millet GP. (2023). Microvascular and oxidative stress responses to acute high-altitude exposure in prematurely born adults. *Sci Rep* **13,** 6860.
2. Manferdelli G, Narang BJ, Bourdillon N, Debevec T & Millet GP. (2023). Physiological Responses to Exercise in Hypoxia in Preterm Adults: Convective and Diffusive Limitations in the O_2 Transport. *Med Sci Sports Exerc* **55,** 482-496.
3. Manferdelli G, Narang BJ, Bourdillon N, Giardini G, Debevec T & Millet GP. Changes in baroreflex sensitivity differ in hypoxia but not in hypercapnia between healthy preterm and term born adults. *J Appl Physiol* (submitted).
4. Manferdelli G, Narang BJ, Bourdillon N, Giardini G, Debevec T & Millet GP. Impaired cerebrovascular CO_2 reactivity at high-altitude in prematurely born adults. *J Physiol* (in revision).
5. Chambion-Diaz M, Manferdelli G, Narang BJ, Giardini G, Debevec T, Pialoux V & Millet GP. Oxidative stress and nitric oxide metabolism responses during prolonged high-altitude exposure in preterm born adults. *Free Radic Biol Med* (submitted).

Publications that are part of the research project

1. Narang BJ, Manferdelli G, Bourdillon N, Millet GP & Debevec T. (2023). Ventilatory responses to independent and combined hypoxia, hypercapnia and hypobaria in healthy pre-term born adults. *J Physiol* (submitted).

2. Manferdelli G, Narang BJ, Debevec T & Millet GP. (2023). Comment on "The Effect of Preterm Birth on Maximal Aerobic Exercise Capacity and Lung Function in Healthy Adults: A Systematic Review and Meta-Analysis". *Sports Medicine* **53,** 1281-1283.
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1. Burtscher J, Citherlet T, Camacho-Cardenosa A, Camacho-Cardenosa M, Raberin A, Krumm B, Hohenauer E, Egg M, Lichtblau M, Müller J, Rybnikova EA, Gatterer H, Debevec T, Baillieul S, Manferdelli G, Behrendt T, Schega L, Ehrenreich H, Millet GP,

Gassmann M, Schwarzer C, Glazachev O, Girard O, Lalande S, Hamlin M, Samaja M, Hüfner K, Burtscher M, Panza G & Mallet R. Mechanisms underlying the health benefits of intermittent hypoxia conditioning. *J Physiol* (submitted).

2. Raberin A, Manferdelli G, Schorderet F, Bourdillon N & Millet GP. Respiratory system limitations to maximal aerobic exercise in normoxia and hypoxia: effects of training and sex. *Chest* (submitted).
3. Manferdelli G, Bourdillon N, Millet GP & Raberin A. Pulmonary function in normobaric hypoxia: Do curves matter? *Am J Physiol Lung Cell Mol Physiol* (submitted).
4. Manferdelli G, Barstow TJ & Millet GP. (2023). NIRS-Based Muscle Oxygenation Is Suitable for Computation of the Convective and Diffusive Components of O_2 Transport at ṾO2max. *Med Sci Sports Exerc* (published ahead of print).
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Abstract

Global premature birth rates have increased considerably across the last three decades, such that 5-18% of all deliveries now occur before the 37th week of gestation. Rising evidence demonstrates underdeveloped and/or compromised respiratory and cardiovascular functions, as well as reduced exercise capacity in preterm born adults. As these physiological systems play central roles in successful high-altitude adaptation, it is not surprising that the potential effects of prematurity on altitude/hypoxia adaptation are of substantial clinical interest.

The main purpose of the present research project was to investigate the physiological responses to acute and prolonged exposure to high-altitude at rest and during exercise in prematurely born, but otherwise healthy, adults. More specifically, this thesis intends to elucidate the mechanisms underlying the cardio- and cerebrovascular, respiratory, muscular, and hematological responses to hypoxia in preterm individuals compared to term born peers.

At rest, we demonstrated normal ventilatory response to normoxia and hypoxia, but blunted microvascular responsiveness, cerebrovascular vasodilation, as well as greater acute oxidative stress level and higher skeletal muscle oxidative capacity. However, during prolonged hypoxic exposure, preterm adults seem to better cope with hypoxia-induced oxidative stress compared to their peers born at term.

During exercise, despite significant convective oxygen transport limitation, preterm adults exhibited a lower susceptibility to the detrimental effects of hypoxia on exercise capacity compared to term born peers, both at cardiopulmonary and cardiovascular level.

In conclusion, despite normal ventilatory responses to hypoxia, prematurely born but otherwise healthy adults present blunted resting cardio- and cerebrovascular responses to acute and prolonged exposure to low oxygen availability. Overall, despite some protective effects while exercising in hypoxia, it cannot be excluded that these individuals may present an increased risk to high-altitude cardiovascular dysfunction and sicknesses.

Résumé

Le taux global des naissances prématurées a considérablement augmenté au cours des trois dernières décennies, aujourd'hui 5 à 18% des accouchements surviennent avant la 37ème semaine de grossesse. Il a été démontré que les fonctions respiratoires, cardiovasculaires et la capacité d'exercice étaient sous développées chez les adultes nés prématurés. Ces systèmes physiologiques jouant un rôle primordial lors de l'adaptation à la haute altitude, les effets d'une naissance prématurée sur l'adaptation à l'altitude sont d'un intérêt significatif.

L'objet principal de ce projet était d'investiguer les réponses physiologiques à l'exposition aiguë et chronique à la haute altitude, au repos et à l'exercice chez des adultes en bonne santé mais nés prématurés. Plus particulièrement, le but était de révéler les mécanismes sous-jacent des réponses à l'hypoxie cardio- et cérébro-vasculaires, respiratoires, musculaires et hématologiques chez des adultes nés prématurés comparés à leurs pairs nés à terme.

Au repos, leur réponse ventilatoire à la normoxie et l'hypoxie était normale, mais leur réponse microvasculaire et leur vasodilatation cérébrovasculaire étaient atténuées et leur stress oxydatif aigu et leur capacité oxydative musculaire étaient augmentés. Toutefois, lors de l'exposition prolongée, les adultes nés prématurés semblaient mieux supporter le stress oxydatif induit par l'hypoxie que leurs pairs.

A l'exercice, malgré une limitation significative du transport convectif de l'oxygène, les adultes nés prématurés étaient moins susceptibles aux effets délétères de l'hypoxie sur la capacité d'exercice comparés à leurs pairs, d'un point de vue cardiopulmonaire et cardiovasculaire.

En conclusion, malgré une réponse ventilatoire à l'hypoxie normale, les adultes nés prématurés présentent des réponses cardio- et cérébro-vasculaires atténuées lors d'une exposition aiguë et chronique à l'hypoxie. Malgré quelque effet protecteur lors de l'exercice en hypoxie, nous ne pouvons exclure que les individus nés prématurés puissent présenter un risque accru de dysfonctionnement cardiovasculaire et de maladie lors de l'exposition à la haute altitude.

List of abbreviations

ADMA
Asymmetric dimethylarginine

AMS
Acute mountain sickness

AOPP
Advanced oxidation protein products

AUC_{2MIN}
Area under the reperfusion curve above baseline TSI until 2 minutes post cuff release

BP
Blood pressure

BPD
Bronchopulmonary dysplasia

BRS
Baroreflex sensitivity

C_aO_2
Arterial oxygen content

CBF
Cerebral blood flow

CO_2
Carbon dioxide

cO_2delivery
Cerebral oxygen delivery

C_vO_2
Venous oxygen content

D_LCO
Lung diffusive capacity for carbon monoxide

DO_2
Diffusive oxygen transport

DmO_2
Skeletal muscle oxygen diffusion capacity

$FEF_{25-75\%}$
Forced expiratory flow between 25 and 75% of total lung volume

FEV_1
Forced expiratory volume in 1 second

F_IO_2
Fraction of inspired oxygen

FRAP
Ferric reducing antioxidant power

GPx
Glutathione peroxidase

HAPE
High-altitude pulmonary edema

Hb
Hemoglobin

HHx
Hypobaric hypoxia

HHx+clamp
Hypobaric hypoxia with $P_{ET}CO_2$ clamped at NNx value

HIF-1α
Hypoxia-inducible factor-1 alpha

HNx
Hypobaric normoxia

$HNx+CO_2$
Hypobaric normoxia with 3% carbon dioxide

HPV
Hypoxic pulmonary vasoconstriction

HRV
Heart rate variability

HVD
Hypoxic ventilatory decline

HVR
Hypoxic ventilatory response

LV
Left ventricle

MAP
Mean arterial pressure

MCA*v*
Middle cerebral artery velocity

MDA
Malondialdehyde

MPO
Myeloperoxidase

$m\dot{V}O_2$
muscle oxygen consumption

N_2
Nitrogen

NIRS
Near-infrared spectroscopy

NNx
Normobaric normoxia

$NNx+CO_2$
Normobaric normoxia with 3% carbon dioxide

NO
Nitric oxide

NO_2^-
Nitrite

NOx
Total nitrite and nitrate

O_2
Oxygen

$P_{cap}O_2$
Capillary partial pressure of oxygen

P_aCO_2
Arterial partial pressure of carbon dioxide

P_aO_2
Arterial partial pressure of oxygen

PEF
Peak expiratory flow

$P_{ET}CO_2$
End-tidal carbon dioxide partial pressure

PGC-1α
proliferator-activated receptor gamma coactivator 1 alpha

P_IO_2
Inspired partial pressure of oxygen

$P_{mito}O_2$
Mitochondrial partial pressure of oxygen

P_B
Barometric pressure

$\dot{Q}$
Cardiac output

$\dot{Q}O_2$
Convective oxygen transport

ROS
Reactive oxygen species

RV
Right ventricle

S_aO_2
Arterial oxygen saturation

SOD
Superoxide dismutase

S_pO_2
Pulse oxygen saturation

$t_{baseline}$
Time required for the TSI signal to return to baseline values

TSI
Tissue saturation index

VA
Alveolar volume

$\dot{V}_E$
Pulmonary ventilation

VEGF
Vascular endothelial growth factor

$\dot{V}O_{2max}$
Maximal oxygen consumption

$\dot{V}O_{2peak}$
Peak oxygen consumption

VOT
Vascular occlusion test

XO
Xantine oxidase

1. Introduction

Introduction

Premature birth, defined as any birth occurring before the 37th week of gestation, represents a growing global concern as it affects over 10% of all live births worldwide. In particular, those occurring "very" preterm (≤32 weeks gestation) are often associated with significant respiratory (Bolton *et al.*, 2015; Simpson *et al.*, 2015; Duke & Lovering, 2020), cardiac (Lewandowski *et al.*, 2013a; Lewandowski *et al.*, 2013b; Haraldsdottir *et al.*, 2019; Telles *et al.*, 2020; Schuermans & Lewandowski, 2022), and vascular challenges (Laurie *et al.*, 2018; Markopoulou *et al.*, 2019; Flahault *et al.*, 2020a; Engan *et al.*, 2021) which likely persist into adulthood. Thanks to advances in perinatal care, there is an ever-increasing number of preterm birth survivors reaching adulthood and a concomitant growing scientific interest in the long-term sequelae of prematurity.

However, rising evidence demonstrates underdeveloped and/or compromised respiratory and cardiovascular functions (Schuermans & Lewandowski, 2022), as well as reduced exercise capacity (Farrell *et al.*, 2015) in preterm born adults. As these physiological systems play central roles in successful high-altitude adaptation, it is not surprising that the potential effects of prematurity on altitude/hypoxia adaptation are of substantial clinical interest. Yet, the scientific community only recently started to systematically assess preterm birth-specific adaptations to hypoxia in humans, that are often (co)modulated by physical activity.

Preterm individuals have been shown to exhibit a blunted hypoxic ventilatory response (HVR) at rest (Bates *et al.*, 2014; Debevec *et al.*, 2019) that may be due to carotid body dysfunction induced by perinatal hyperoxic treatment (Bates *et al.*, 2014). While direct evidence is lacking, the above-mentioned impairments on several systems, combined with systemic vascular dysfunction, might augment susceptibility to high-altitude illnesses, particularly acute mountain sickness (AMS). However, exercise performed at moderate intensity was shown to override the reduced resting HVR, suggesting this population to well tolerate exercise

performed in hypoxia (Debevec *et al.*, 2019). Despite this, the long-term impact of premature birth on high-altitude adaptation capacity and/or on resting and exercise physiological responses during acute and prolonged exposure to hypoxia is still uncertain. Few studies showed reduced (Debevec *et al.*, 2019) or unchanged (Farrell *et al.*, 2015) power output at volition exhaustion in this population compared to their term born peers, as well as no clear influence of premature birth on gas exchange efficiency (Duke *et al.*, 2014), interstitial lung water accumulation (Debevec *et al.*, 2022b), and pulmonary vascular responses (Laurie *et al.*, 2018) during exercise in hypoxia. Furthermore, premature birth seems to influence redox balance modulation and nitric oxide pathway, both primarily involved in the ventilatory and vascular responses to hypoxia (Debevec *et al.*, 2017). Collectively, whilst most of the prematurity-specific adaptations may seem unfavorable for efficient altitude adaptation, observations such as lower oxidative stress responses (Martin *et al.*, 2018) and lower reduction in aerobic power (Farrell *et al.*, 2015) under hypoxia might indicate that premature birth and/or perinatal management could, at least in some aspects, induce a 'preconditioning' effect which may reduce the detrimental impact of hypoxia on several systems.

Accordingly, the present research project aimed to investigate the physiological responses to acute and prolonged exposure to high-altitude at rest and during exercise in prematurely born, but otherwise healthy, adults. Specifically, this thesis intends to elucidate the mechanisms underlying the cardio- and cerebro-vascular, respiratory, muscular, cognitive, and hematological responses to hypoxia in preterm individuals compared to term born peers.

2. Review of the literature

2.1 Premature birth

Global premature birth rates are continuing to increase such that 5-18% of all deliveries now occur before the 37th week of gestation (Liu *et al.*, 2016). Preterm birth occurs during a critical period during which an important development of several organs, tissues, and vascular network occurs (**Figure 1**). Understanding the role and the underlying mechanisms of altered organogenesis and angiogenesis may help targeting preventive and therapeutic strategies to minimize the onset and progression of several chronic diseases typically observed in preterm born adults. Indeed, recent remarkable progresses in modern perinatal care have reduced birth-related complications, with survival rates now often exceeding 95% (GBD Mortality and Causes of Death Collaborators, 2016). These epidemiological trends have resulted in a growing

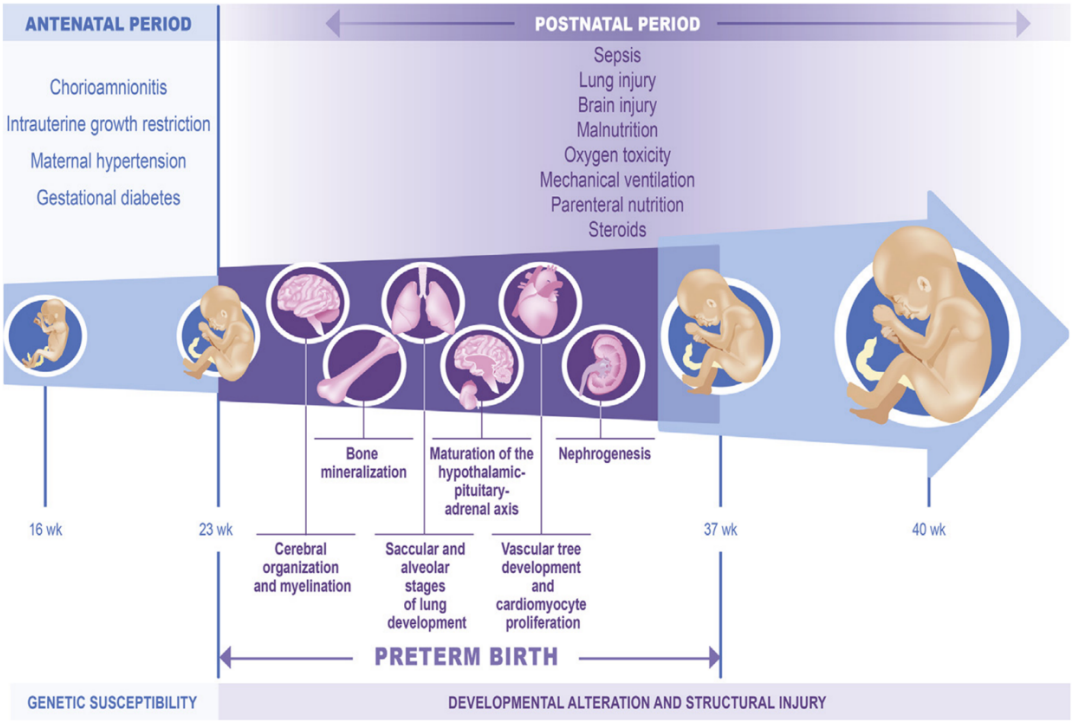

Figure 1. Schematic representation of fetal development. Premature birth, and especially extremely (≤28 weeks gestation) and very (≤32 weeks gestation) preterm birth, occurs at critical stages of organ and vascular development, thus hampering normal maturation of several systems. After birth, complications related to prematurity *per se* - and perinatal treatments (e.g., O_2 supplementation therapy, steroids) - further alter normal development of organs and tissues. From Luu *et al.* (2016).

number of premature birth survivors, with a large proportion now reaching adulthood. However, those born before 32 weeks gestation have increased risk for several respiratory, cardiac, and vascular sequelae which persist into adulthood (Lewandowski *et al.*, 2020; Duke *et al.*, 2022). As young adults, they have a 3-fold higher risk for systemic hypertension (Crump *et al.*, 2011; Hovi *et al.*, 2016), a 5-fold higher risk for pulmonary hypertension (Naumburg *et al.*, 2015; Goss *et al.*, 2018a; Goss *et al.*, 2019), and up to 17-fold higher risk for heart failure compared to term-born peers with a clear dose-response relative to the degree of prematurity (Carr *et al.*, 2017). This translates into impairments in daily life activities and overall quality of life, evidenced by lower quality of life scores and higher exercise intolerance, as extensively described by a growing number of investigations (Vrijlandt *et al.*, 2006; Lovering *et al.*, 2013; Duke *et al.*, 2014; Farrell *et al.*, 2015; Caskey *et al.*, 2016; Haraldsdottir *et al.*, 2018b; Debevec *et al.*, 2019). Accordingly, research exploring the long-term effects of premature birth beyond adolescence and maturity is a critical and contemporary area of research. The respiratory (pulmonary mechanics, airways structure and function), cardiopulmonary (gas exchange, pulmonary hemodynamics), and cardiovascular (heart size and dimension, arterial circulation) consequences of premature birth have been well characterized over the last few years (El Mazloum *et al.*, 2014; Bolton *et al.*, 2015; Carr *et al.*, 2017; Raju *et al.*, 2017; Goss *et al.*, 2018a; Crump *et al.*, 2019; Dance, 2020; Flahault *et al.*, 2020a; Telles *et al.*, 2020; Engan *et al.*, 2021). Nevertheless, many of the underlying physiological and pathophysiological factors underlying these impairments, and their clinical implications, are just beginning to be understood. However, our current understanding of prematurity-related long-term health consequences is primarily derived from investigations of extremely (≤28 weeks gestation) and very preterm born individuals, with little data available in individuals born moderate-to-late preterm (**Figure 2)**.

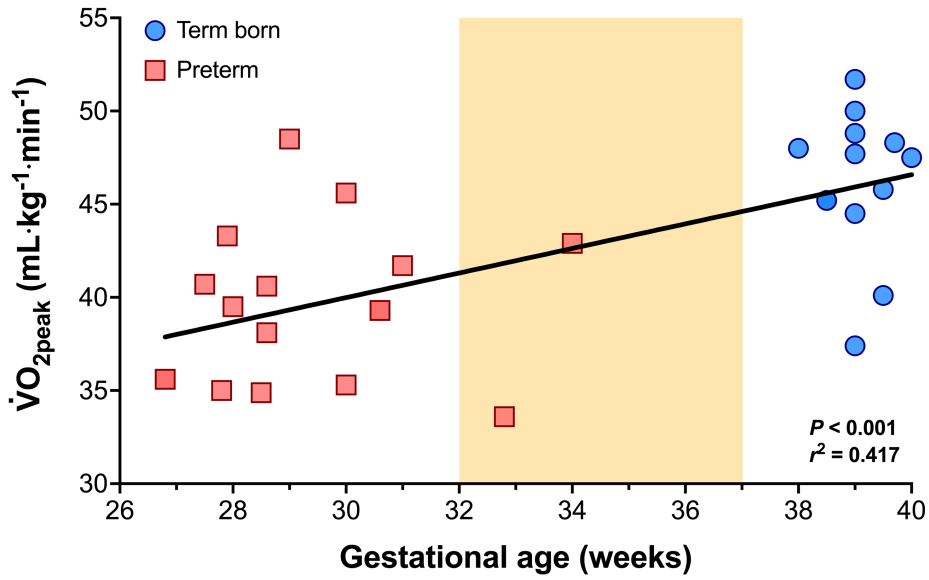

Figure 2. Peak oxygen uptake ($\dot{V}o_{2peak}$) vs. gestational age reported by previous studies investigating exercise capacity in prematurely born (red squares) and term born age-matched adults (blue dots). The black line depicts the linear relationship between these parameters. The yellow shaded area between gestational age 32 and 37 weeks represents the region where little is known about peak oxygen consumption ($\dot{V}o_{2max}$). Data from Vrijlandt *et al.* (2006); Lovering *et al.* (2013); Duke *et al.* (2014); Farrell *et al.* (2015); Caskey *et al.* (2016); Goss *et al.* (2018a); Vrijlandt *et al.* (2018); Debevec *et al.* (2019); (Haraldsdottir *et al.*, 2019); Haraldsdottir *et al.* (2020); Huckstep *et al.* (2021); McKay *et al.* (2021); O'Dea *et al.* (2021). Modified from Duke and Lovering (2020).

2.1.1 Pulmonary system and gas exchange

The pulmonary system is responsible for moving oxygen (O_2) from the ambient air to venous blood and removal of carbon dioxide (CO_2) from the venous blood to the external environmental (West & Luks, 2020). This system presents a great structural and functional plasticity – i.e., changes in neural control in response to various stimuli (i.e., hypoxia, hyperoxia) – both during neonatal development and in adulthood (Carroll, 2003; Fuller & Mitchell, 2017). However, immediately after birth (postnatal maturation period) there is critical time window during which environmental factors can irreversibly modulate structural and functional properties of the respiratory system (Carroll, 2003; Reeves & Gozal, 2005). This window is particularly relevant to understand the long-term sequelae of premature birth on the respiratory system, and the consequent ventilatory response to hypoxia and hypercapnia. Due

to their immature respiratory system (**Figure 1**), premature newborns are often exposed to elevate O_2 concentrations for a few weeks immediately after birth in the attempt to maintain arterial O_2 saturation above 85% (Mohamed *et al.*, 2020b). However, prolonged exposure to hyperoxia during neonatal period disrupts the normal development of the peripheral chemoreceptors located in the carotid bodies, leading to reduced ventilatory response to sustained hypoxia and hyperoxia (Erickson *et al.*, 1998; Donnelly *et al.*, 2005; Wenninger *et al.*, 2006; Bates *et al.*, 2014) (**Figure 3**).

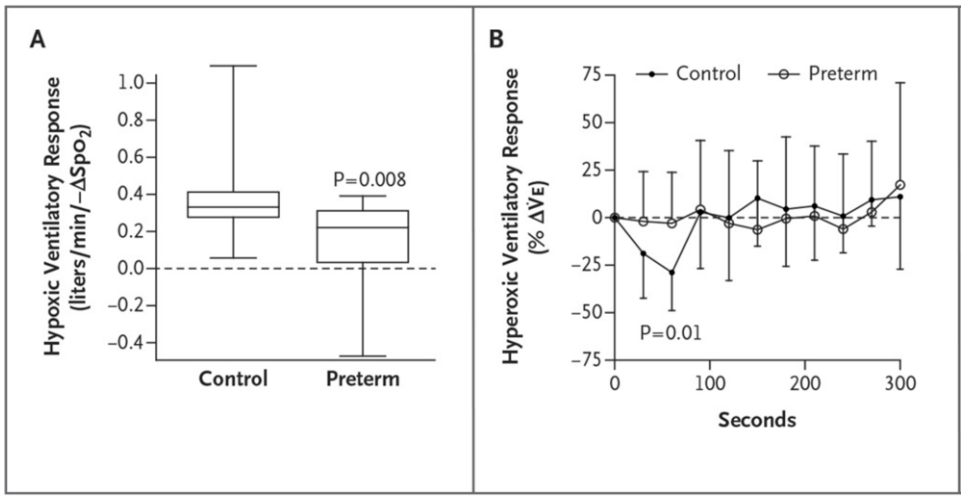

Figure 3. Blunted ventilatory response to two 5-minute hypoxic (fraction of inspired O_2 - F_IO_2 - = 12%; left panel) and hyperoxic (F_IO_2 = 100%; right panel) exposures in young prematurely born adults compared to age-matched term born peers. Of note, all the preterm participants included in the study underwent O_2 supplementation therapy at birth. From Bates *et al.* (2014).

In addition to carotid chemoreceptors disruption, neonatal exposure to hyperoxia is the key mechanism involved in the development of bronchopulmonary dysplasia (BPD). Despite continuous and remarkable improvements in perinatal care of preterm newborns, BPD incidence remains at ~40% for preterm infants born at or before 28 weeks gestation (Stoll *et al.*, 2015). BPD is a chronic lung disease characterized by arrested lung development, blunted alveolarization, respiratory muscle hypertrophy, altered pulmonary vascular and microvascular function, as well as right ventricular hypertrophy secondary to pulmonary hypertension

(Baraldi & Filippone, 2007; Islam *et al.*, 2015; Lignelli *et al.*, 2019). Emerging reports also suggest a new pattern of pulmonary dysfunction in very preterm individuals, called "new BPD", which is characterized by less severe respiratory distress (Ronkainen *et al.*, 2015; Duke *et al.*, 2022), though the risk for later onset of respiratory diseases and lung function abnormalities remains elevated (Martinez, 2016; Morrow *et al.*, 2017). However, to better understand the effects of premature birth *per se* beyond maturation in healthy adults, it is important to separate preterm with history of BPD from those without reports of this lung disease. Indeed, even in absence of BPD, preterm newborns still present variable degrees of airways obstruction, lower residual volumes, and decreased lung compliance which persist during adulthood (Islam *et al.*, 2015) and increase the risk to develop cardiopulmonary disorders such as chronic obstructive cardiopulmonary disease (Bui *et al.*, 2018).

Moreover, independently of history of BPD, recent imaging works demonstrated that almost 50% of very and extremely preterm born individuals present an exaggerated resting pulmonary vascular pressure (average pulmonary artery pressure of 20 mmHg) during childhood (Zivanovic *et al.*, 2017) and young adulthood (Goss *et al.*, 2018a) (**Figure 4**), and therefore often cross the cut-off for pulmonary hypertension diagnosis. Of note, even in case of normal pulmonary artery pressure at rest, preterm individuals have a greater risk to develop pulmonary hypertension during mild and intense exercise (Goss *et al.*, 2018a; Laurie *et al.*, 2018). The mechanisms underlying the rise in pulmonary artery pressure at rest remain unclear, though a recent large survey-based study indicated premature birth *per se*, neonatal O_2 supplementation therapy, and early hospitalization for respiratory illness as risk predictors for future development of pulmonary hypertension during adulthood (Goss *et al.*, 2019). The increased pulmonary artery pressure in individuals born prematurely represent a significant challenge during adulthood, leading to RV dysfunction and early onset of cardiovascular diseases.

Several meta-analyses and original studies confirm the persistence of pulmonary injuries in

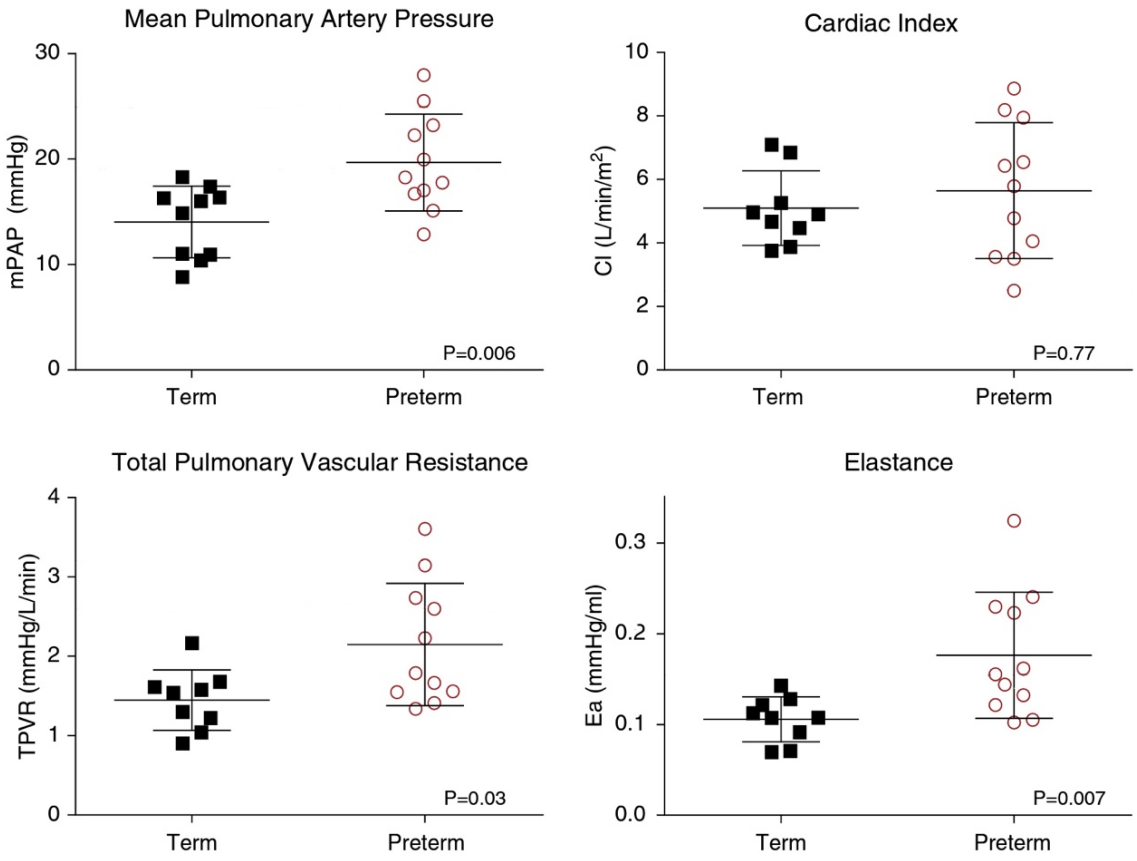

Figure 4. Mean pulmonary artery pressure (mPAP), cardiac index (CI), total pulmonary vascular resistance (TPVR), and pulmonary elastance (Ea – representative of total right ventricular afterload) obtained at rest in term born and preterm adults. mPAP was measured by catheter positioned in the pulmonary artery, while CI as the ratio between cardiac output, measured using the direct Fick method, and body surface area. TPVR and Ea were calculated as the ration between mPAP and cardiac output, and between mPAP and stroke volume, respectively. From Goss *et al.* (2018a).

adults born preterm, including lower pulmonary function (Vrijlandt *et al.*, 2006; Simpson *et al.*, 2015; Caskey *et al.*, 2016; Molgat-Seon *et al.*, 2019), and resting gas transfer (Narang *et al.*, 2009), abnormal chest radiographs (Aukland *et al.*, 2006), and obstructive lung disease (Narang, 2010), though recent works reported normal pulmonary function and lung diffusion capacity (Farrell *et al.*, 2015; Goss *et al.*, 2018a; Debevec *et al.*, 2019) in this cohort which might be related to improvements in neonatal therapies at the beginning of the millennium (Vollsaeter *et al.*, 2015). Heliox-O_2 gas mixture is often used to lessen airflow resistance and alleviate pulmonary mechanical constrains, thus to improve lung function (Babb, 2001).

However, when used in preterm adults with reduced spirometry indices and obstructive

pulmonary profile, heliox-O_2 air did not improve lung function (assessed as the shape of the maximum expiratory flow-volume curve) (Molgat-Seon *et al.*, 2019). This suggest that, in adult survivors of premature birth, both mechanical and structural factors within the airways contribute to impaired lung function.

Altogether, these structural and functional impairments within the pulmonary system may lead to ventilation-to-perfusion mismatch, diffusion limitations, and ultimately limit exercise capacity in preterm adults.

2.1.2 Cardiovascular system

The cardiovascular system is composed by the heart, which must pump the blood into the systemic and pulmonary circulation, and the vessels (arteries, veins, and capillaries), which must transport oxygenated (arterial) and deoxygenated (venous) blood to peripheral tissues (O_2 extraction) and to the alveoli (gas exchange), respectively (**Figure 5**).

Premature birth occurs during a key period of cardiovascular development (**Figure 1**), leading to an early physiological shift to a relatively "hyperoxic" environment with increased systemic and decreased pulmonary vascular resistance (Lewandowski *et al.*, 2020). As a result, growing evidence in animals and humans describes a specific cardiovascular phenotype related to prematurity that likely contributes to short- and long-term risk of various diseases, such as hypertension, heart failure, and ischemic heart disease (Telles *et al.*, 2020).

Recent meta-analyses and reviews summarizing existing findings on cardiac differences between preterm and term born individuals demonstrated changes in morphology and function of the premature heart, which were evident across all developmental stages, including neonatal life, infancy, childhood, and into young adulthood (Telles *et al.*, 2020; Schuermans & Lewandowski, 2022). Imaging data (by cardiovascular magnetic resonance) showed that, independently of body size and blood pressure, individual born preterm, compared to age-

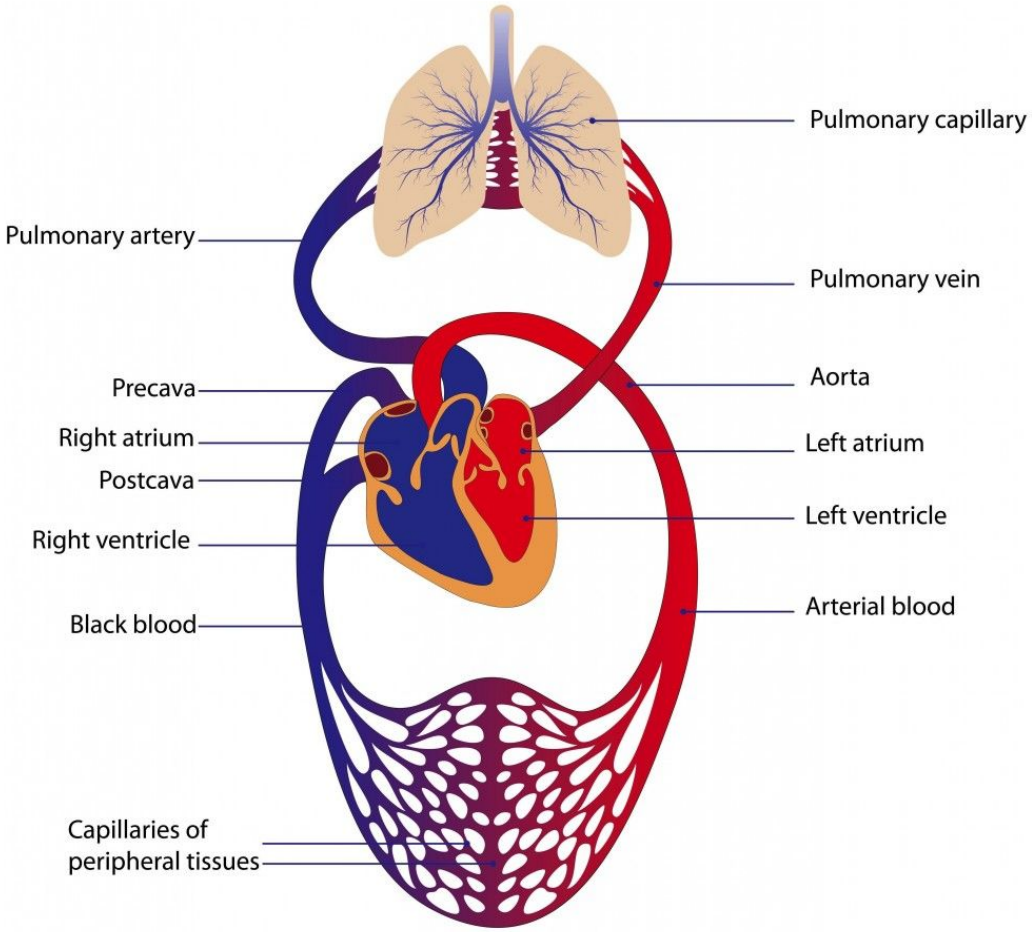

Figure 5. Simplistic representation of the cardiovascular system.

matched term born peers, present greater left ventricular (LV) wall thickness, smaller internal LV cavity dimensions and lengths, the latter being the main contributor to reduced LV end-diastolic volumes at rest (Lewandowski *et al.*, 2013a). Moreover, in the same data set, reduced longitudinal myocardial deformation – assessed as lower longitudinal strain and strain rate, as well as diastolic strain rate – was observed in preterm adults compared to term born controls, despite similar LV ejection fractions (Lewandowski *et al.*, 2013a). Reduced LV chamber size and end-diastolic volumes, and impaired longitudinal systolic strain were also confirmed using echocardiography in a large cohort of normotensive young adults born prematurely compared to peers born at term (Huckstep *et al.*, 2018). Similar to the left side, specific cardiac morphology and function have been described for the right ventricle (RV) of preterm born individuals. Lower RV end-diastolic volumes (independently of body size), reduced RV

ejection fraction (with 6% of young adults having values below the clinical normal range of 45% for ejection fraction), as well as lower RV systolic and diastolic strain and strain rates were reported during adolescence and young adulthood in preterm compared to term born individuals using either cardiac magnetic resonance imaging or echocardiography (Lewandowski *et al.*, 2013b; Mohamed *et al.*, 2020a). Moreover, at the level of the RV, preterm adults present a unique 3-dimensional geometry, with reduced global systolic function assessed by RV fractional area of change and tricuspid annular plane systolic excursion (Mohamed *et al.*, 2020a). Inconsistent findings also suggest different LV and RV mass in preterm adolescents and young adults, with either greater (Lewandowski *et al.*, 2013a; Lewandowski *et al.*, 2013b; Bassareo *et al.*, 2016; Huckstep *et al.*, 2018; Mohamed *et al.*, 2020a) or lower (Kowalski *et al.*, 2016; Goss *et al.*, 2020; Harris *et al.*, 2020) ventricular mass compared to term born individuals. The reduced cardiac mass seems to be particularly evident in those who were born before 32 weeks of completed gestation, period around which there is an important increase in the number of binucleated cardiomyocytes (Kim *et al.*, 1992).

Preterm individuals are also susceptible to develop hypertension during young adulthood (Alexander & Intapad, 2012; Bertagnolli *et al.*, 2016). Reduced aortic diameter, narrowed and stiffer arteries, and restricted vascular bed are potential candidates for increased risk of high blood pressure in this population (Lewandowski *et al.*, 2013a). However, while strong evidence demonstrates reduced ascending and descending aortic lumen in adults born preterm (Bonamy *et al.*, 2005; Boardman *et al.*, 2016; Kowalski *et al.*, 2016), data on conduit arterial stiffness (assessed non-invasively via pulse wave velocity) are inconsistent, with higher (Boardman *et al.*, 2016; Barnard *et al.*, 2020b) or similar (Kowalski *et al.*, 2016) pulse wave velocity in major conduit arteries. This discrepancy might be due to different gestational age of the preterm participants across the studies, as well as to a higher prevalence of overweight/obesity in the investigated preterm cohorts compared to the control groups. Indeed, preterm individuals are

more likely to be obese (Mathai *et al.*, 2013; Gnawali, 2021), which is a known factor associated with increased arterial stiffness during adulthood (Zebekakis *et al.*, 2005).

At microvascular levels, evidence in preterm humans is scarse, though an animal model simulating premature birth by neonatal exposure to hyperoxia demonstrated lower systemic microvascular density and *in vitro* angiogenesis in adult rats (Yzydorczyk *et al.*, 2008).

Beyond altered cardiac and vascular growth, structure, and function, the autonomic control of blood pressure may also play a role in the etiology of hypertension and other cardiovascular diseases in the preterm population. Indeed, the development of the autonomic nervous system (e.g., baroreflex sensitivity - BRS, and heart rate variability - HRV) mostly occurs during the third trimester (Van Leeuwen *et al.*, 1999; Andriessen *et al.*, 2005) and is therefore interrupted in very-to-extremely preterm born infants. Limited results suggest autonomic dysfunction in adult survivors of premature birth (Mathewson *et al.*, 2015; Haraldsdottir *et al.*, 2018a; Karvonen *et al.*, 2019) and highlight its contribution in rising the risk of cardiac diseases in this specific group of adults. Among other autonomic regulatory mechanisms, the baroreflex is a powerful homeostatic mechanism that detects changes in blood pressure (BP) and evokes reflex circulatory adjustments aiming to buffer BP changes (La Rovere *et al.*, 1995). The sensitivity of the arterial baroreflex control or cardiac activity plays a pivotal role in human health as demonstrated by the inverse relation between BRS and the risk of mortality after myocardial infarction (La Rovere *et al.*, 1998) or by the improved BRS after endurance training (Parati *et al.*, 2001). Early findings collected in newborns over a wide range of gestational age (27 – 41 weeks) showed a linear increase in BRS with gestational age in infants (**Figure 6**) (Gournay *et al.*, 2002; Javorka *et al.*, 2021a).

The same authors (Gournay *et al.*, 2002) also investigated postnatal maturation of BRS in a subgroup of eight very preterm infants and showed a delayed BRS maturation over 30 days after birth in this group compared to normal *in utero* BRS development of term born newborns.

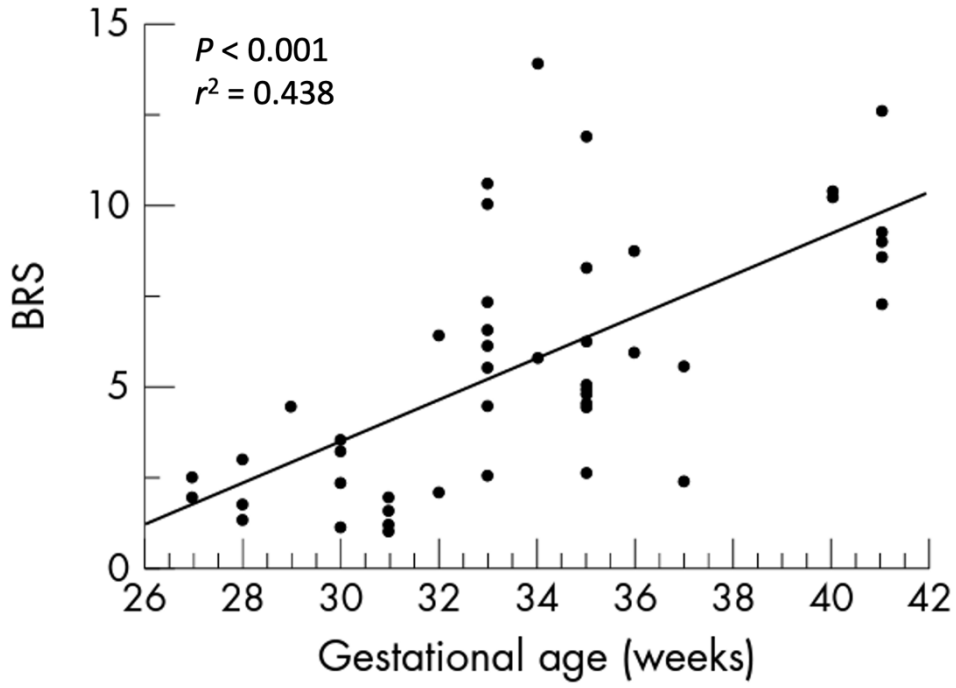

Figure 6. Linear increase in baroreflex sensitivity (BRS) with gestational age in 38 prematurely born and 9 term born infants. From Gournay et al. (2002).

Likewise, previous results suggested underdeveloped cardiac baroreflex in premature infants, which became more functional during the postnatal period (Mazursky *et al.*, 1998). The depressed BRS in preterm newborns was suggested to be associated with decreased cardiac parasympathetic activity assessed by HRV (Eiselt *et al.*, 1993; Javorka *et al.*, 2021b).

Surprisingly, only few studies have investigated the cardiac autonomic control in preterm individuals during adulthood. Reduced HRV indices of cardiac vagal activity (including baroreflex-mediated effects) were observed in young adults born preterm compared to peers born at term (Karvonen *et al.*, 2019). Furthermore, lower BRS was observed in adults with history of very low birth weight (condition typically observed in preterm newborns) (Leotta *et al.*, 2007). Therefore, negative effects of premature birth on the autonomic regulatory control of the cardiac system may persist during adulthood, though additional evidence is required to confirm these limited previous findings.

2.1.3 Cerebrovascular system

Brain function is strongly dependent on a close matching between metabolic demands, appropriate O_2 and nutrients delivery, and removal of waste products of the cerebral metabolism. This matching requires continuous regulation of cerebral blood flow (CBF), which can be summarized into four integrated mechanisms (**Figure 7**): *a*) cerebral autoregulation, which describes the ability of the cerebrovasculature to maintain constant CBF in response to changes in perfusion pressure; *b*) vascular reactivity to vasoactive stimuli, including changes in arterial CO_2 partial pressure (P_aCO_2) and, in severe hypoxia, decreased arterial partial pressure of O_2 (P_aO_2) below 50 mmHg; *c*) neurovascular coupling, described as the CBF response to local changes in neural activity; and *d*) endothelium-dependent responses (Willie *et al.*, 2014b; Claassen *et al.*, 2021).

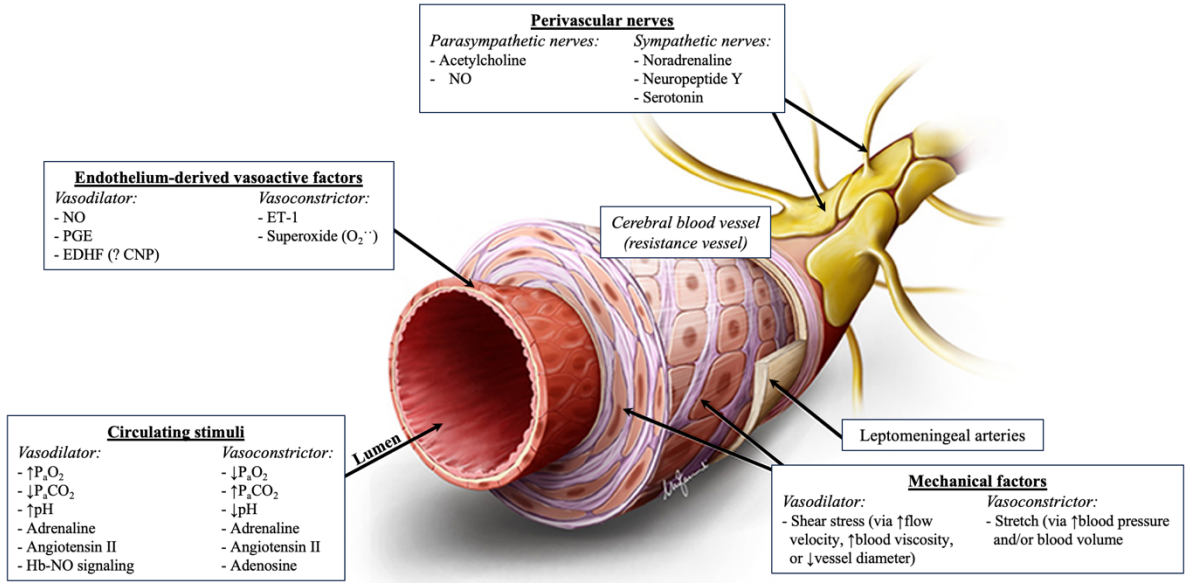

Figure 7. Primary factors regulating the diameter of cerebral vessels, and thus cerebral blood flow. Through myogenic tone (mechanical factors), transmural pressure influences arterial diameter through direct smooth muscle contraction or relaxation. Fluctuations in circulating stimuli, including changes in arterial blood gases, hemoglobin-based nitric oxide (Hb-NO) signaling, and hormonal secretion, lead to cerebral vasoconstriction or dilatation. The endothelium also plays a key role via the secretion of substances that may act directly on smooth muscle cells. Lastly, in the neurogenic response, neurons and glia mediate smooth muscle physiology by releasing various neurotransmitters with vasoactive properties. Note that angiotensin and adrenaline can cause either cerebral vasoconstriction or vasodilation depending on which receptor they bind to (e.g., angiotensin-1 receptors

(vasoconstriction) and angiotension-2 receptors (vasodilation); for adrenaline receptors, vasoconstriction is via the alpha receptors and vasodilation is via the beta receptors). *CNP*, C-natriuretic peptide; *EDHF*, endothelial-derived hyperpolarizing factor; *ET-1*, endothelin-1; *NO*, nitric oxide; P_aCO_2, arterial carbon dioxide partial pressure; *PGE*, prostaglandins; P_aO_2, arterial partial pressure of oxygen. Modified from Ainslie *et al.* (2014).

Altered vascular remodeling and angiogenesis (Brew *et al.*, 2014; Lewandowski *et al.*, 2020), hypertension (Alexander & Intapad, 2012; de Jong *et al.*, 2012; Bertagnolli *et al.*, 2016; Brewer *et al.*, 2023), increased ventilatory sensitivity to hypercapnia (Rigatto *et al.*, 1975; Manferdelli *et al.*, 2021), as well as restricted vascular bed, narrowed and stiffer arteries (Bonamy *et al.*, 2005; Kowalski *et al.*, 2016; Lewandowski *et al.*, 2020) have been well described in preterm adults. Altogether, although experimental evidence on the long-term effects of premature birth is still missing, the functional and anatomical changes associated with preterm delivery may predispose these individuals to incomplete development of the cerebral circulation during childhood, leading to impaired cerebrovascular regulation in adulthood.

Cerebral autoregulation aims to maintain CBF constant despite changes in cerebral perfusion pressure by constantly adjusting cerebrovascular tone (Paulson *et al.*, 1990). In young healthy adults, resting CBF is maintained relatively constant at ~50 $mL \cdot 100\ g_{brain\ tissue}^{-1} \cdot min^{-1}$ (Lassen, 1985) within a range of mean arterial pressure (MAP) between ~±10% from resting values (defined as autoregulation plateau) (Brassard *et al.*, 2021). Above and below the limits of this pressure interval, cerebral autoregulation is lost and changes in CBF are passively dependent on MAP (**Figure 8A**) (Brassard *et al.*, 2021). Although experimental data in preterm adults are missing, findings in preterm newborns suggest that cerebral autoregulation is either absent (Lou *et al.*, 1979; Milligan, 1980) or present only over a narrow range of MAP (van de Bor & Walther, 1991). However, to date, it is still unclear whether cerebral autoregulation becomes normal with maturation in adults born prematurely.

It is also well established that the brain vasculature is extremely sensitive to P_aCO_2 (**Figure 8B**) (Brian *et al.*, 1996). Increased CO_2 level in arterial blood results in a relaxation of the

cerebral vascular smooth muscles (cerebral vasodilation), while a decrease in P_aCO_2 leads to vasoconstriction of cerebral vessels (Wei *et al.*, 1980). Cerebral vascular reactivity to CO_2 is often used to assess cerebrovascular endothelial health (Portegies *et al.*, 2014; Willie *et al.*, 2014b), as it integrates several mechanisms within the cerebral vasculature in response to changes in P_aCO_2 (Ainslie & Duffin, 2009). Cerebrovascular CO_2 reactivity was reported to be either normal (Greisen & Trojaborg, 1987; Pryds *et al.*, 1990; Mosca *et al.*, 1999; Jayasinghe *et al.*, 2003) or increased (Aly *et al.*, 2019) in preterm newborns shortly after birth and undergoing mechanical ventilation. Of note, these studies performed indirect measurements (i.e., brain Near Infrared Spectroscopy - NIRS) to assess cerebral oxygenation changes in response to spontaneous end-tidal CO_2 ($P_{ET}CO_2$) fluctuations. Surprisingly, data on cerebrovascular regulation to changes in arterial blood gases (P_aCO_2 and P_aO_2) in adults born prematurely are still missing.

Thigh CBF regulation is also influenced by neuronal activation, mechanisms referred as neurovascular coupling. Briefly, fluctuations in the neuronal activity cause changes in local blood flow mediated by vascular smooth muscles through astrocyte-mediated activation

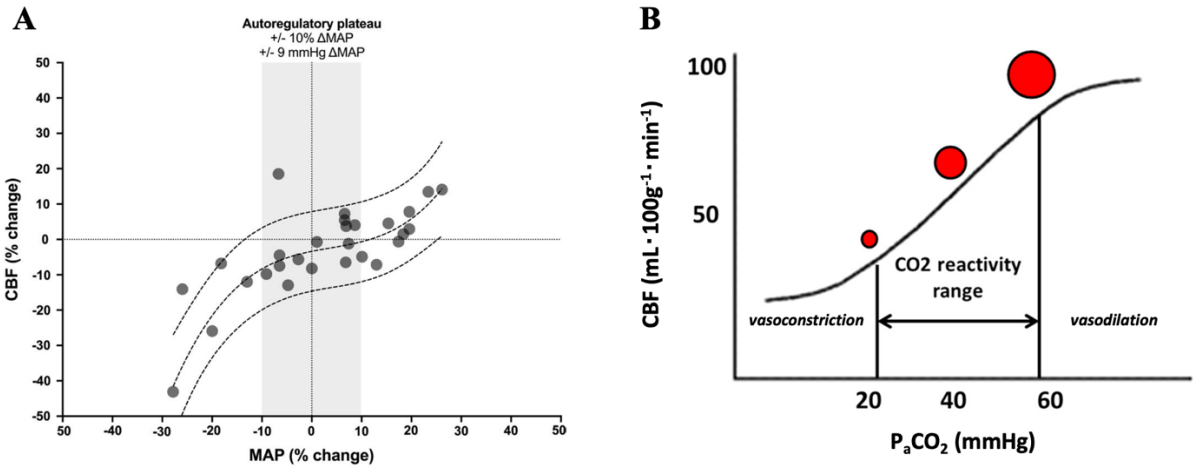

Figure 8. Relationship between spontaneous relative changes in MAP from baseline (%ΔMAP) and concomitant relative changes in cerebral blood flow (CBF) when MAP decreases and increases (panel A), and between absolute changes in CBF in response to changes in arterial carbon dioxide partial pressure (P_aCO_2; panel B). In the right panel, the red circles represent the changes in diameter of the cerebral vessels in response to changes in P_aCO_2. Panel A from Brassard et al. (2021) and panel B modified from Godoy et al. (2021).

(Phillips *et al.*, 2016). Research on the effects of premature birth on neurovascular coupling is limited and inconsistent in newborns, while evidence is missing during adulthood. Preliminary findings suggest that the coupling between cerebral hemodynamic response to cortical activation is likely developed in preterm infants born after 32 weeks gestation (Greisen *et al.*, 1985), while this regulatory mechanisms was shown to be absent during spontaneous neuronal activation (assessed by NIRS) in extremely preterm newborn (Wong *et al.*, 2009). Interestingly, in the same cohort of extremely preterm infants, neurovascular coupling was restored by infusion of dopamine (Wong *et al.*, 2009).

Overall, premature birth seems to affect all the regulatory mechanisms of CBF, leading to an increased risk for stroke events (Crump *et al.*, 2021). Surprisingly, data on cerebrovascular regulation during adulthood in this growing high-risk population are still missing.

2.1.4 Skeletal muscle system

Decades of research in skeletal muscle physiology have highlighted the complexity of this system, which is designed to accomplish multiple tasks, including generating contraction, glucose homeostasis, and help maintain venous return and thus $\dot{Q}$. While early research investigating the effects of premature birth in infants and adults traditionally focused on the pulmonary, cardiac, and vascular consequences in this population, less attention was given to the skeletal muscle system. Neonatal exposure to hyperoxia, with consequent increases in oxidative stress and reactive O_2 species (ROS) production, may induce changes in skeletal muscle structure (i.e., capillarization, fiber type composition) and function (Deprez *et al.*, 2021); thereby possibly contributing to the onset and progression of musculoskeletal diseases and reduced exercise capacity in prematurely born individuals. However, existing findings are primarily based on animal models using postnatal exposure to hyperoxia to simulate premature birth (Tetri *et al.*, 2018). Yet, small evidence of impaired mitochondrial capacity also comes

from muscle biopsies collected postmortem in preterm neonates (Wenchich *et al.*, 2002; Honzik *et al.*, 2008), while no study investigated skeletal muscle function in human preterm adults.

A recent animal model demonstrated greater fat-to-lean mass ratio, increased muscle fatigability, and lower skeletal muscle mitochondrial oxidative capacity in adult rats exposed to 14 days of postnatal hyperoxia (to simulate premature birth) compared to controls who were not exposed to high O_2 levels (Tetri *et al.*, 2018). Interestingly, these impairments compared to the control group were not present in female rats undergoing the same hyperoxic protocol (Tetri *et al.*, 2018). A later study, also conducted in male and female rat exposed to 7 days postnatal hyperoxia, showed increased ROS formation, greater inflammation within the skeletal muscles, muscle atrophy, fiber type shifting (from type I slow fibers to type IIb fast-fatigable fibers), and overall impaired skeletal muscle function (Deprez *et al.*, 2021). Importantly, these alterations in skeletal muscle function were evident in both juvenile and adult rats, and, similarly to (Tetri *et al.*, 2018), the effects were larger in males compared to females (Deprez *et al.*, 2021). These findings in rats were confirmed in extremely preterm neonates, where mitochondrial energy metabolism and oxidative capacity were significantly reduced (Wenchich *et al.*, 2002; Honzik *et al.*, 2008).

2.1.5 Oxidative stress and nitric oxide pathway

Oxidative stress is characterized by an homeostatic imbalance between pro-oxidant and antioxidant enzyme activity, in favor of the oxidants (Sies & Jones, 2007), though growing knowledge rather suggests a disruption at the level of redox signaling and control (Jones, 2006). Mitochondria are the primary drivers increasing oxidative stress by producing ROS through oxidative phosphorylation (Halliwell, 2022). Continuous advancements on the multiple effects of ROS in humans have highlighted new roles of ROS in both physiological and pathological processes (D'Autreaux & Toledano, 2007). At the cellular level, ROS regulate growth,

apoptosis, and other signaling processes, while, at systemic level, they contribute to blood pressure regulation, cognitive and immune functions (Brieger *et al.*, 2012; Halliwell, 2022). On the other hand, chronically elevated ROS concentrations may lead to non-specific damage to proteins, lipids, and nucleic acids, often inducing irreversible functional alterations implicated in the onset and progression of several diseases (Brieger *et al.*, 2012) (**Figure 9**).

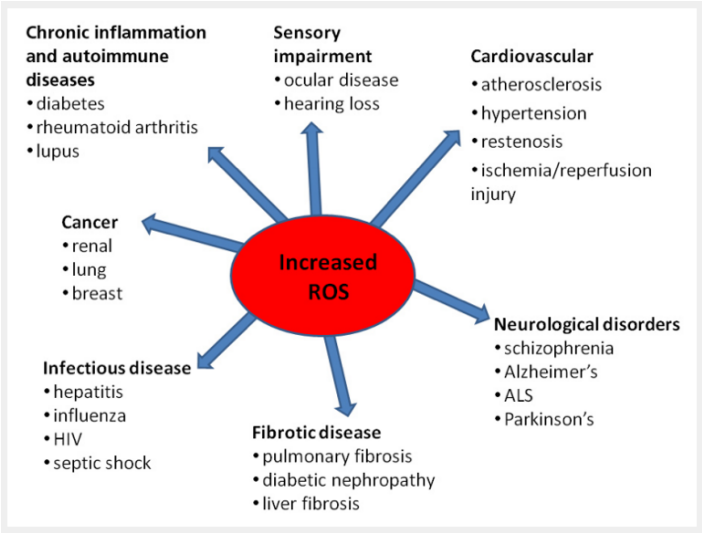

Figure 9. Contribution of increased reactive oxygen species (ROS) production on the onset and progression of several diseases. From Brieger *et al.* (2012).

Several stimuli promote oxidative stress, including premature birth (Martin *et al.*, 2018; Moore *et al.*, 2018) and neonatal O_2 supplementation therapy (Millán *et al.*, 2018; Burtscher *et al.*, 2022). This is also supported by consistent findings on the inverse relationship between gestation age and increased oxidative stress levels (Comporti *et al.*, 2004; Negi *et al.*, 2012). Preterm newborn abruptly leaves the hypoxic intrauterine environment (P_aO_2 = 20 - 25 mmHg) and must cope with an "O_2-enriched" ambient air (P_aO_2 = 100 mmHg - normoxia) despite immature pulmonary system and lack of antioxidant defenses. Furthermore, the majority of premature infants undergo pure O_2 supplementation therapy immediately after birth, which further augments oxidative stress via increased ROS production (Moore *et al.*, 2018). Importantly, greater ROS formation seems to persist during maturation in this population, as

higher oxidative stress was recently described in premature adolescents (Filippone *et al.*, 2012), while inconsistent results were observed in adults born preterm. Indeed, systemic oxidative stress biomarkers were higher (e.g., 8-isoprostane in exhaled breath condensate, total superoxide dismutase (SOD) and glutathione peroxidase (GPX) in blood) (Martin *et al.*, 2018) or similar (e.g., advanced oxidation protein products (AOPP), Catalase and SOD in plasma) (Flahault *et al.*, 2020b) in healthy preterm adults compared to term born peers. Although little is known about the mechanisms underlying the greater level of oxidative stress markers in this cohort, increased oxidative stress levels in the placenta of the mother during pregnancy (Wu *et al.*, 2015), lower antioxidant enzymatic and non-enzymatic activity (Abdel Ghany *et al.*, 2016), and perinatal exposure to hyperoxia (Vento *et al.*, 2009) represent suitable candidates driving the exaggerated oxidative stress in preterm survivors (**Figure 10**) (Martin *et al.*, 2018).

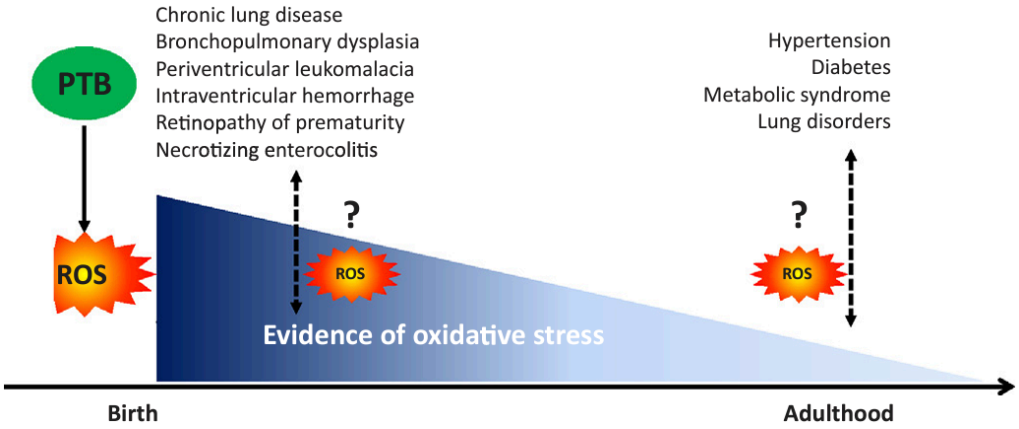

Figure 10. The chicken and egg paradigm of oxidative stress in preterm individuals from birth to adulthood. Premature birth (PTB) increases the risk to develop several diseases at birth and up to adulthood. The higher oxidative stress during the life of PTB survivors could play a critical role in the pathogenesis of these diseases, but it could also be induced by these diseases. *ROS*, reactive oxygen species. From Martin *et al.* (2018).

Nitric oxide (NO) is a key signaling molecule involved in a variety of biological function, including vascular tone regulation both rest and under conditions of increased blood flow in response to skeletal muscle contraction or increased shear stress. At local level, together with other local vasoactive mediators, NO has a potent vasodilatory effect on the microvasculature,

thereby directly regulating overall vascular tone and blood flow to match tissue perfusion and metabolism (Melikian *et al.*, 2009).

However, in several diseases and inflammatory states, NO production within the vasculature increases considerably and, together with other ROS molecules, contributes to oxidative stress (Beckman & Koppenol, 1996). Likewise, reduced NO levels, via asymmetric dimethylarginine (ADMA)-induced inhibition of NO synthesis, are also associated with multiple morbidities characterized by endothelial dysfunction (Boger *et al.*, 1998; Boger, 2003; Ueda *et al.*, 2007). Early findings suggest increased plasma ADMA levels in premature newborn during the first four weeks of life (Vida *et al.*, 2009), suggesting early endothelial dysfunction which may persist into adulthood and explain the increased risk for cardiovascular disease after maturation in this population. These findings, although limited, were extended to young adults born extremely-to-very prematurely (Bassareo *et al.*, 2012), demonstrating the persistence of impaired vascular regulation in preterm adult survivors. Recent ultrasonographic assessment of major arterial vessels in a large cohort of young preterm and term born adults confirmed increased stiffness of both the carotid and brachial arteries in preterm participants compared to term born controls, despite similar serum NO levels (Flahault *et al.*, 2020a). Of note, experimental animal data suggest a direct link between neonatal exposure to hyperoxia and reduced endothelium-dependent vasodilation (Yzydorczyk *et al.*, 2008) secondary to impaired nitric oxide synthase function and regulation (Yzydorczyk *et al.*, 2013), though these findings were not confirmed in humans (Flahault *et al.*, 2020a). Therefore, additional work is required to understand the vascular phenotype, and the role of NO, in preterm survivors and potential therapeutic targets to improve endothelial function during adulthood in this population.

2.1.6 Exercise capacity

Exercise capacity is the result of an integrative and systemic response involving several systems and hematological responses within the human body (Wagner, 1988; Levine, 2008; Wagner, 2022). Considering the current state of art presented in the previous sections, it is not surprising that exercise capacity and cardiorespiratory fitness (i.e., $\dot{V}O_{2peak}$) have been almost univocally demonstrated to be reduced in this population (Vrijlandt *et al.*, 2006; Lovering *et al.*, 2013; Duke *et al.*, 2014; Farrell *et al.*, 2015; Haraldsdottir *et al.*, 2018b; Debevec *et al.*, 2019), even with similar physical activity levels (Tikanmaki *et al.*, 2017). However, due to the continuing improvement of neonatal care treatments and likely the heterogeneity of this population (i.e., lifestyle, socioeconomic status, presence of BPD), the sites of functional limitation to exercise capacity in adult survivors of premature birth remain debated.

The interest on exercise capacity in prematurely born adults began in the early 2000, when Vrijlandt and colleagues firstly reported impaired lung diffusion capacity, airway obstruction, and reduced exercise capacity in young adults born preterm, though the authors excluded a causal relationship between poor lung function and exercise intolerance (Vrijlandt *et al.*, 2006). In later studies, exercise capacity was univocally found lower in preterm adults, despite either normal (Farrell *et al.*, 2015; Debevec *et al.*, 2019) or reduced (Duke *et al.*, 2014; Lovering *et al.*, 2014; Caskey *et al.*, 2016; Duke *et al.*, 2019) pulmonary function in preterm participants compared to term born controls. Novel findings also demonstrated that, in adults born preterm with reduced aerobic exercise capacity, breathing a heliox-enriched gas mixture improved time-to-exhaustion (Duke *et al.*, 2019). Therefore, these authors concluded of a potential role of mechanical respiratory constraints in limiting exercise capacity in this population. Of note, most of these studies who attributed exercise intolerance in preterm adults to poor lung function also reported concomitant history of BPD in the investigated preterm cohort. Recent data from a Scandinavian group suggest greater pulmonary function (assessed via spirometry) in 11-yr-

old children born preterm at the turn of the millennium compared with those born in the early 1990s (Vollsaeter *et al.*, 2015), likely due to improvements in neonatal respiratory management. Intriguingly, lung function indices were similar between 11-yr-old term born controls and age-matched preterm born children in the late 1990s-early 2000s (Vollsaeter *et al.*, 2015). Therefore, in absence of BPD, other mechanisms involved the O_2 cascade and downstream pulmonary function are likely to determine reduced exercise capacity in prematurely born adults.

Emerging evidence suggests adverse cardiac development and impaired cardiac reserve as primary contributors to early exercise cessation in these individuals (Haraldsdottir *et al.*, 2019; Macdonald *et al.*, 2021; Schuermans & Lewandowski, 2022; Yang *et al.*, 2022). Indeed, young adults born preterm have smaller biventricular cardiac chamber sizes and lower cardiac mass (Goss *et al.*, 2020), with reduced stroke volume reserve, and thus cardiac output ($\dot{Q}$), during submaximal and maximal exercise (**Figure 11**) (Goss *et al.*, 2018b; Huckstep *et al.*, 2018; Haraldsdottir *et al.*, 2020). This impaired stroke volume reserve is also supported by the lack of correlation between the absolute stroke volume increase from rest to peak exercise intensity and $\dot{V}O_{2peak}$ during incremental upright cycling exercise in the preterm cohort but not in the control group (**Figure 12**) (Haraldsdottir *et al.*, 2020).

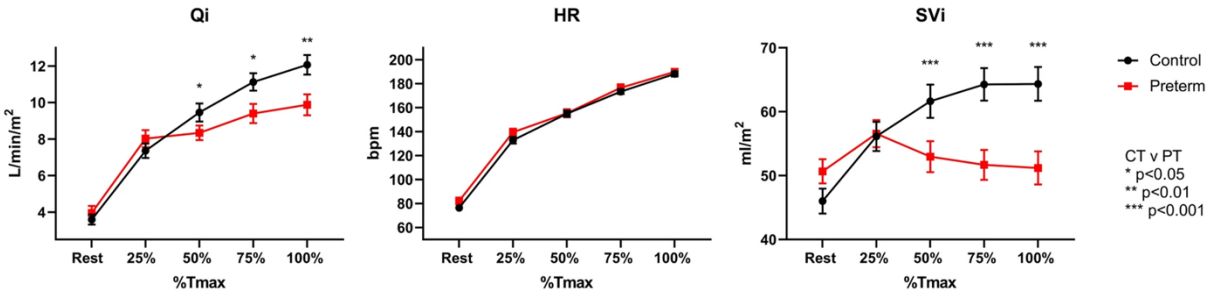

Figure 11. Cardiac hemodynamic response to incremental upright cycling exercise to exhaustion in term born (black dots) and age-matched preterm (red squares) adolescents. *HR*, heart rate; *Qi*, cardiac output indexed to body size; *SVi*, stroke volume indexed to body size; *%Tmax*, percentage of total time of exercise. Modified from Haraldsdottir *et al.* (2020).

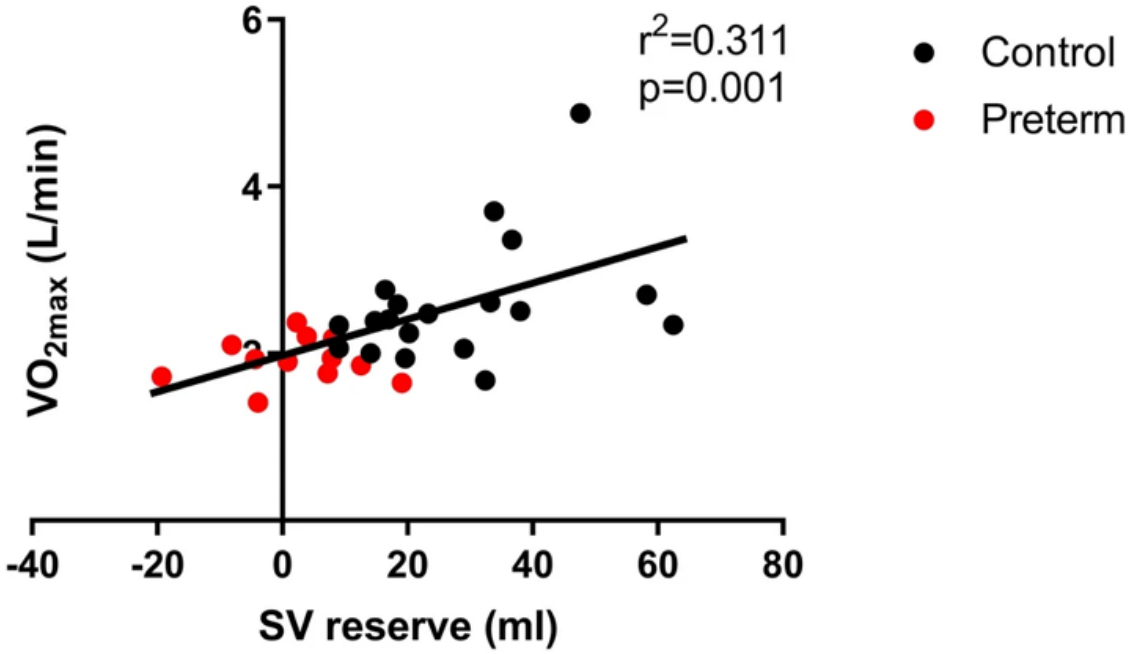

Figure 12. Linear relationship between stroke volume (SV) reserve (i.e., the change in SV from rest to peak exercise intensity) and maximal oxygen consumption ($\dot{V}O_{2max}$) in term born (black dots), but not in preterm (red dots), adolescents. From Haraldsdottir *et al.* (2020).

Of note, the intrinsic and autonomic cardiac control of heart rate seems to be unaffected by premature birth, as demonstrated by similar kinetics and maximal heart rate during incremental exercise test in preterm and term born individuals (**Figure 11**) (Haraldsdottir *et al.*, 2020). On the contrary, heart rate recovery at the first and/or second minute following maximal exercise was slower in young adults born extremely preterm compared to age-matched controls (Haraldsdottir *et al.*, 2019; Huckstep *et al.*, 2021), therefore suggesting some mechanisms of autonomic dysfunction to be potentially unmasked by high-intensity exercise in these individuals.

Moreover, invasive studies suggest exaggerated increases in both vascular resistance, particularly within the pulmonary artery (Goss *et al.*, 2018b; Laurie *et al.*, 2018), and in systemic blood pressure (Barnard *et al.*, 2020a) with exercise in preterm adults. These defective responses are likely to result from decreased vascular density and/or increased arterial stiffness (Goss *et al.*, 2018b; Barton *et al.*, 2021), and are expected to independently impair the cardiac response to exercise. Over time, this exaggerated pressor response further exacerbates cardiac

remodeling. This is confirmed by a recent study that demonstrated more robust LV hypertrophic remodeling in preterm born adults with systemic hypertension relative to hypertensive term born adults (Mohamed *et al.*, 2021); suggesting a preterm myocardium that is more sensitive to afterload. This greater sensitivity to afterload was also shown in the RV, so that acute reduction in RV afterload using sildenafil (a pulmonary vasodilator) resulted in an improvement in RV intraventricular flow and overall $\dot{Q}$ (Corrado *et al.*, 2021). However, to date, it remains unknown which ventricle primarily drives exercise limitations in adults born preterm, and to what extent an exaggerated rise in afterload affects exercise limitations via impaired cardiac function.

The final step in the O_2 cascade from ambient air to mitochondria is O_2 diffusion at skeletal muscle level. According to the Fick Principle,

$$\dot{V}O_2 = \dot{Q} \times a - vO_2diff \qquad (1)$$

where a-vO_2diff represents O_2 extraction in the active skeletal muscles during exercise. Current research on the long-term effects of premature birth on skeletal muscle contractile function, energetics, and O_2 extraction, as well as their contribution to exercise intolerance, remains scarse. Lower skeletal muscle contractility in both upper and lower limbs was reported in adolescents born preterm (Rogers *et al.*, 2005). Furthermore, increased O_2 extraction (assessed non-invasively as greater NIRS-derived deoxygenated hemoglobin) was recently described in 5-years old children born preterm, compared with age-matched participants born at term, during submaximal exercise (6-minute walking test) (Owen-Jones *et al.*, 2020). Keeping in mind the lower stroke volume reserve in preterm adults, one may argue that increased O_2 extraction at skeletal muscle level represents a compensatory mechanism to counterbalance central limitations to exercise, at least in the moderate intensity domain (**Figure 13**). However, as already highlighted above (see Chapter 2.1.4 - *Skeletal muscle*), animal models using postnatal O_2 supplementation therapy to simulate premature birth demonstrated long lasting effects of

hyperoxia on skeletal muscle composition, morphology, function, and oxidative capacity (Tetri *et al.*, 2018; Deprez *et al.*, 2021). Therefore, future studies should address the contribution of skeletal muscle to exercise capacity – or incapacity – in adult survivors of premature birth.

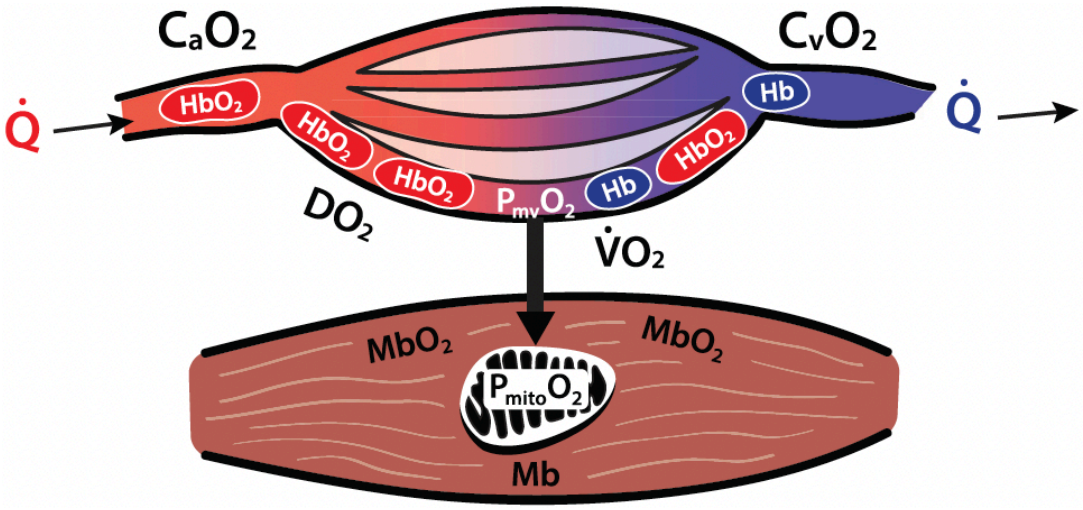

Figure 13. Schematic representation showing components of oxygen delivery and oxygen diffusion from capillary to muscle fiber mitochondria. In individuals born preterm, the reduced cardiac output ($\dot{Q}$), and therefore skeletal muscle blood flow, during submaximal exercise might be counterbalanced by increased oxygen extraction at skeletal muscle level. *CaO_2*, arterial oxygen content; *CvO_2*, venous oxygen content draining the muscle/limb; *DO_2*, diffusivity of oxygen; *Mb*, myoglobin; *$PmitoO_2$*, mitochondrial partial pressure of oxygen; *$PmvO_2$*, microvascular partial pressure of oxygen; *$\dot{V}O_2$*, oxygen uptake. From Barstow (2019).

2.1.7 Sex differences

Sex represents a vital biological variable which influences many physiological mechanisms and almost all aspects of health and disease. However, many research studies still investigate only males or do not consider sex differences. Accordingly, prematurity-related research has only recently highlighted sex as an important variable driving specific short- and long-term sequelae and physiological responses at rest and during exercise in the preterm population.

Latest findings from a large national study on preterm newborns indicated male sex as an independent risk factor for major morbidities, especially in extremely preterm babies and adults (Shim *et al.*, 2017). The importance to investigate sex difference in preterm survivors was also

confirmed by a series of later studies where males, compared to females, showed larger cortical volumes during childhood (Skiold *et al.*, 2014; Benavides *et al.*, 2019), increased risk for adverse respiratory illness later in life (Kotecha *et al.*, 2018), as well as higher skeletal muscle fatigability, lower mitochondrial oxidative capacity in skeletal muscle, more mitochondrial damage, and higher glycolytic enzyme expression (Tetri *et al.*, 2018). In contrast, cardiac LV and RV dimensions and functions at rest seem to be similarly impaired in preterm males and females compared to term born peers, though RV size is lower in preterm males only, when compared to term born individuals (Goss *et al.*, 2020).

2.2 Physiological responses to hypoxia

In moving into highlands above 2500 m, whether transiently or permanently, humans have relied upon an array of successful responses to hypoxia that range across the physiological continuum from organs to cells and genome. Many physiological changes occur in humans reaching high-altitude, including a reduction in exercise capacity, increased ventilation, polycythemia, and sleep disturbances. Since the first studies on the effects of high-altitude on humans in the late 1800s from Paul Bert (West, 2016; Richalet, 2021) , several studies have been conducted in some of the most elevated regions on Earth to understand altitude physiology. Unravelling what transpires with successful and unsuccessful adaptation to acute and chronic hypoxia is inextricably linked to better understanding of human physiology and pathophysiology.

The term “hypoxia” refers to a any partial pressure of O_2 in the inspired air ($P_IO_2 = F_IO_2 \times$ barometric pressure) below the normoxic value of 150 mmHg (Conkin & Wessel, 2008). Consequently, the lower P_IO_2 reduces the O_2 diffusion from the alveoli to the systemic circulation and lately tissue O_2 delivery at peripheral level (**Figure 14)**.

It is critical to distinguish hypobaric hypoxia – where environmental hypoxia results from decreases in barometric pressure (P_B) with no changes in F_IO_2 (i.e., high-altitude) – and normobaric hypoxia – resulting from reduced F_IO_2 without affecting P_B (i.e., simulated altitude, hypoxic chamber). The physiological effects of hypobaric vs. normobaric hypoxia are still debated (Girard *et al.*, 2012; Millet *et al.*, 2012a, b, 2013; Millet & Debevec, 2020a, b). Overall, while the magnitude of the response might differ, both hypobaric and normobaric hypoxia stimulate specific responses by different systems in the body (**Figure 15**), which attempt to mitigate the negative effects of the fall in P_aO_2 and to ensure adequate O_2 supply to the brain and active tissues.

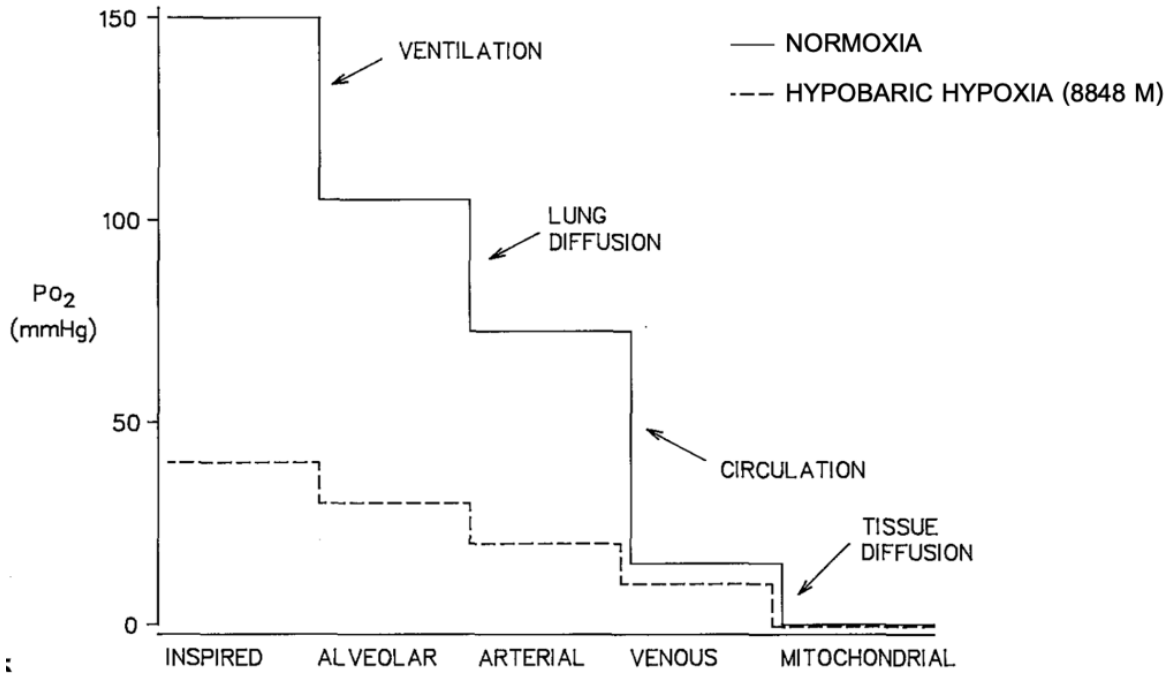

Figure 14. Oxygen (O_2) cascade showing the fall in oxygen partial pressure (PO_2) from inspired air (~150 mmHg) to skeletal muscle mitochondria (~2 - 3 mmHg) at near maximal exercise performed at sea-level (solid line) and in extreme normobaric hypoxia (at an inspired PO_2 ~59 mmHg, equivalent of the summit of Mt. Everest - broken line). The vertical lines represent the pressure gradient in O_2 at each level of the O_2 cascade. This gradient is significantly reduced in conditions of hypoxia. Modified from Reeves *et al.* (1987).

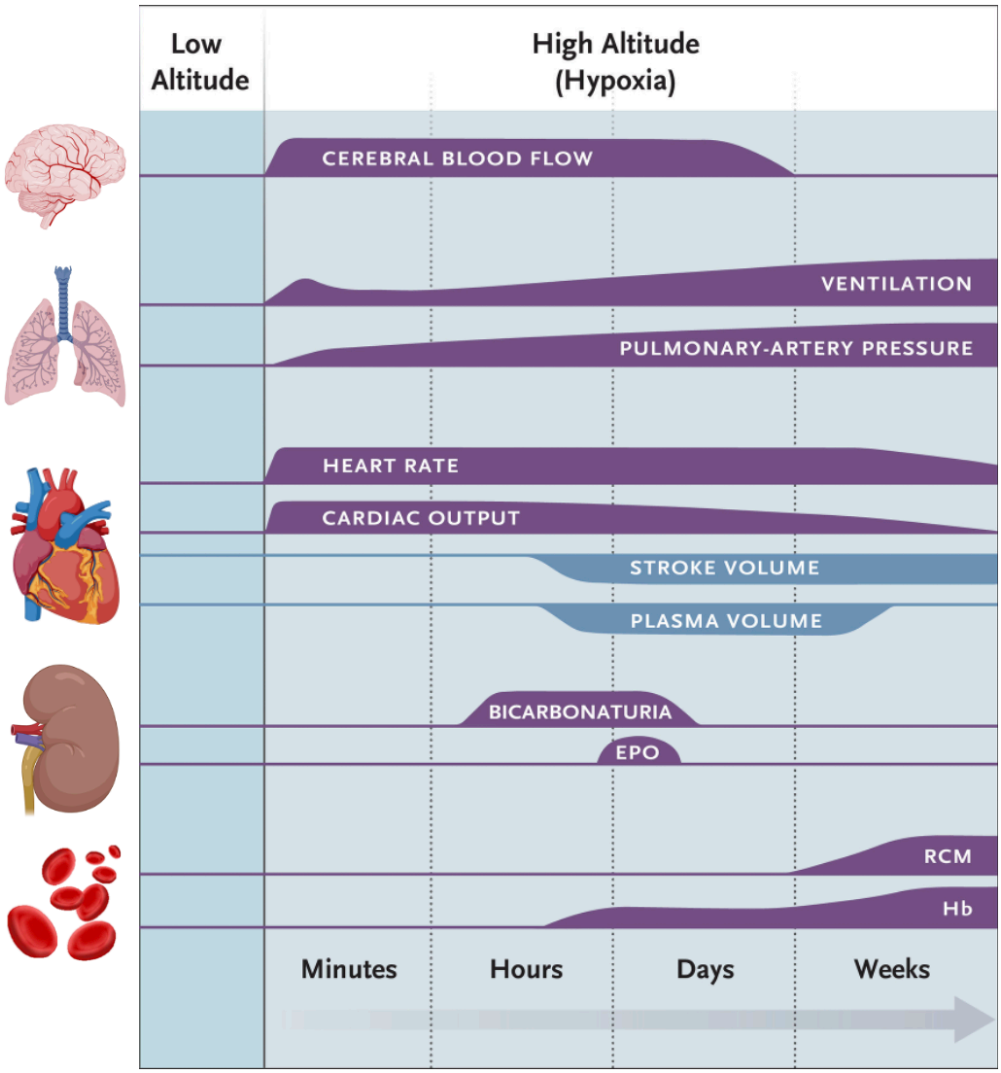

Figure 15. Time course of the major physiological responses to hypoxia ranging from minutes and hours (acute response) to days and weeks (acclimatization) of exposure. Brain, lungs, heart, kidneys, and blood are primarily involved in the response to hypoxia. Parameters that increase from sea-level to high-altitude are shown in purple, while those which decrease are displayed in blue. The pattern and timing of the responses are similar among persons, but the magnitude of the responses can vary markedly. *EPO*, erythropoietin; *RCM*, red-cell mass; *Hb*, hemoglobin. Modified from Luks and Hackett (2022).

However, independently of whether is hypobaric or normobaric hypoxia, it is crucial to consider the changes occurring in the O_2 cascade (**Figure 14**), from the inhaled air to the mitochondria at peripheral level where O_2 is ultimately consumed to generate adenosine triphosphate molecules through aerobic metabolic processes (West *et al.*, 2012). However, as far as advantageous this process is, it does not restore performance to the same level as in normoxia. The time courses of these responses vary, though most of the changes take place

over the first days of exposure and up to few weeks (**Figure 15**). Also, the response of the body to hypoxia depends crucially on the rate, as well as the degree, of hypoxia, with high interindividual variability. For the purpose of this thesis, the following sections will discuss the physiological changes occurring during acute and chronic (up to few weeks) exposure to hypoxia. These responses are triggered by the stimulation of peripheral chemoreceptor located in the carotid bodies (**Figure 16**). Especially, although peripheral chemoreceptors stimulation affects several organs in the body, the majority of changes over the first hours of permanence in hypoxia occur at the level of the respiratory and cardiovascular systems (**Figure 17**).

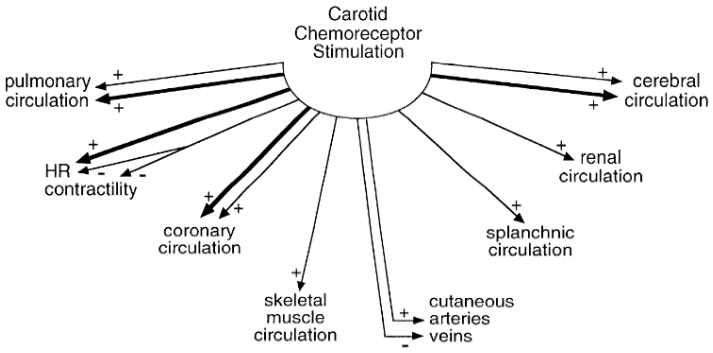

Figure 16. Hypoxemia is sensed by peripheral chemoreceptors localized in the carotid bodies. Their activation induces several systemic responses. *HR*, heart rate. From Marshall (1994).

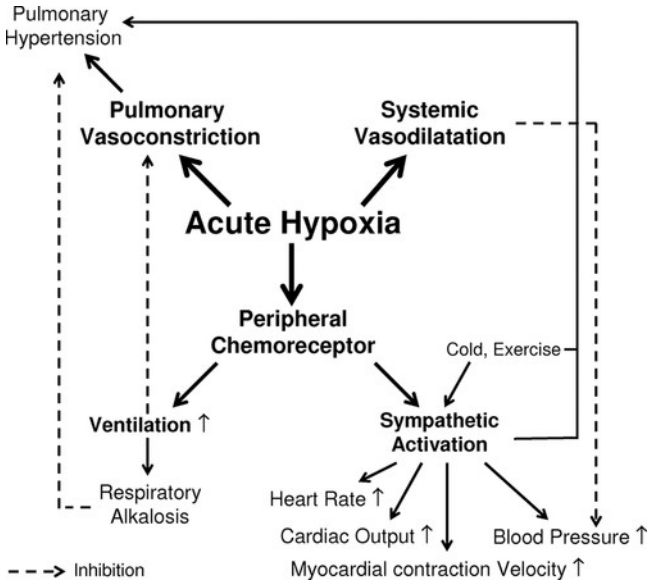

Figure 17. Schematic representation of the effects of short-term hypoxia on the respiratory and cardiovascular systems. From Bartsch and Gibbs (2007).

2.2.1 Respiratory system

As the first step in the O_2 transport cascade, the respiratory system is crucial in the immediate responses to hypoxia. Important changes occur in both pulmonary mechanics and gas exchange that, in turn, affect hematological parameters, including arterial blood gas balance and acid–base status, and clinical outcomes, such as exercise tolerance and development of acute altitude illnesses.

Within 10 minutes of exposure to hypoxia, an abrupt increase in $\dot{V}_E$, termed hypoxic ventilatory response (HVR), occurs in response to a decrease in P_aO_2 (i.e., hypoxemia). This acute hyperventilatory response, which remains elevated for a few days after arrival at high-altitude or entering a hypoxic chamber (**Figure 15**), typically occurs when the P_IO_2 drops below 100 mmHg (corresponding to ~3000 m) (Rahn & Otis, 1949). Functionally, it attempts to maintain a functional alveolar-to-arterial O_2 pressure gradient and consequently to reduce the alteration in O_2 pulmonary diffusion (West & Luks, 2020). The acute ventilatory increase in hypoxia is primarily stimulated by specialized O_2-sensitive cells (chemoreceptors) located the carotid and aortic bodies (though the latter plays only a secondary role), which sense hypoxemia and increase their afferent activity (**Figure 18**) (Gonzalez *et al.*, 1994). The afferent input travels to the brain stem where further processing by the respiratory centers takes place and lead to increase ventilatory drive (Teppema & Dahan, 2010; West *et al.*, 2012). Furthermore, at more severe levels of hypoxemia (P_aO_2 below 50 mmHg), central chemoreceptors situates in the medulla are also involved in the control of breathing, leading to a further increase in $\dot{V}_E$ (Willie *et al.*, 2014b). Intriguingly, the relationship between the rise in $\dot{V}_E$ and the decrease in P_aO_2 is not linear, but rather hyperbolic (**Figure 19A**), likely due to the reciprocal effects $\dot{V}_E$ on P_aCO_2 and vice versa.

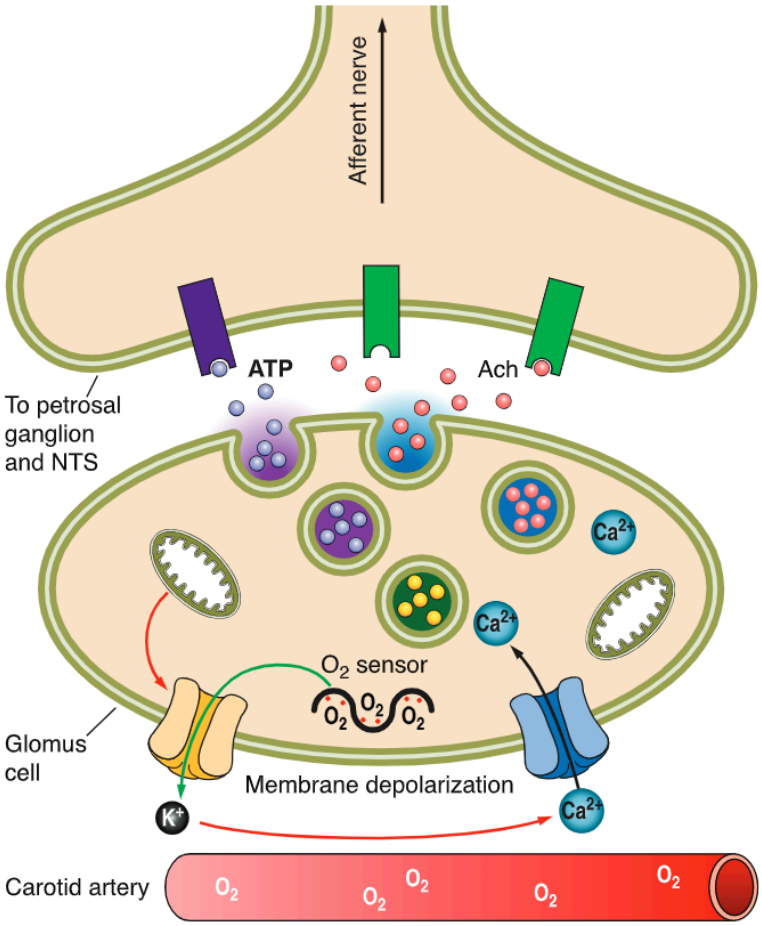

Figure 18. Schematic representations of the transduction cascade in O_2-sensing cells located in the carotid bodies that occurs in conditions of acute hypoxia. Briefly, a reduction in O_2 tension in the arterial blood flowing in the carotid artery is detected by primary O_2 sensors type I carotid body cells, which rapidly communicate with potassium (K^+) channels leading to a closure of these latter. In turn, via membrane depolarization and increases in intracellular calcium (Ca^{2+}) concentration, the release of neurotransmitters (i.e., acetylcholine and adenosine triphosphate) lead to excitation of the afferent nerve that run in the carotid nerve sinus up to the respiratory centers in the brain stem. *Ach*, acetylcholine; *ATP*, adenosine triphosphate; *NTS*, nucleus of the solitary tract. From Teppema and Dahan (2010).

The P_aCO_2-$\dot{V}_E$ relationship is central in understanding the magnitude of the ventilatory response to hypoxia. In conditions of uncontrolled P_aCO_2 (i.e., poikilocapnic hypoxia), the hypoxia-induced hyperventilation causes a drop in P_aCO_2, which, in turn, decreases the breathing drive and masks the full HVR. The time course of changes in $\dot{V}_E$ during exposure to hypoxia consists of three phases (**Figure 19B**) (Smith *et al.*, 2001; Ainslie *et al.*, 2013):

I. Upon initial exposure to hypoxia and up to about 5-10 minutes there is a marked increase in $\dot{V}_E$, and consequent fall in P_aCO_2.

II. This is followed by a so-called hypoxic ventilatory decline (HVD), consisting of a rapid decrease in $\dot{V}_E$ which persists up to 20 - 30 minutes after initial hypoxic exposure. The exact mechanisms behind the HVD are still debated but seems to be related to decreased peripheral chemoreceptor sensitivity (Dahan *et al.*, 1996; Duffin & Mahamed, 2003), elevations in CBF (Hoiland *et al.*, 2015), and/or neural stimulus (Pamenter & Powell, 2016), rather than direct effect of the reduced P_aCO_2. Of note, the magnitude of HVD is proportional to the size of the initial hypoxic-induced hyperventilatory response (Georgopoulos *et al.*, 1989; Dahan *et al.*, 1996; Kimura *et al.*, 1998).

III. From 30 minutes from initial hypoxic exposure to about two weeks sojourning at a given altitude, $\dot{V}_E$ keeps rising with consequent decreases in P_aCO_2. This phase (ventilatory acclimatization) is driven by an increase in HVR, changes in CO_2 chemosensitivity, and, perhaps, some direct effects of hypoxia on the central nervous system.

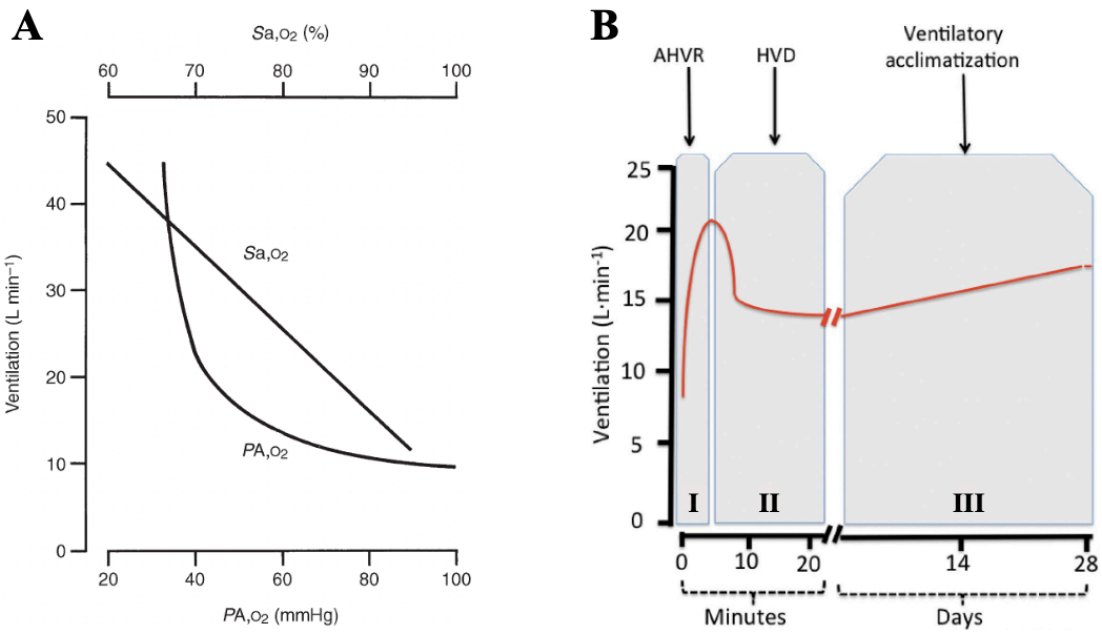

Figure 19. Change in pulmonary ventilation in response to changes in arterial oxygen partial pressure or arterial oxygen saturation (Panel A), and time course of the change in ventilation over 20 minutes upon exposure and up to 28 days of sojourn in hypoxia (Panel B). Note that the relationship between ventilation and arterial oxygen partial pressure ($PA,_{O2}$) is exponential, while it is linear between ventilation and arterial oxygen saturation ($Sa,_{O2}$). *AHVR*, acute hypoxic ventilatory response; *HVD*, hypoxic ventilatory decline. Panel A from West *et al.* (2012), Panel B modified from Ainslie *et al.* (2013).

It is also important to highlight that, although the three phases of ventilatory response to hypoxia are present in each individual, the magnitude of ventilatory increase presents a great interindividual variability (Vizek *et al.*, 1987), with independent effects of age, sex, training status, and respiratory diseases (Richalet *et al.*, 2012; Richalet *et al.*, 2020; Mallet *et al.*, 2023). Current methodologies to assess hypoxic chemosensitivity, and its potential role in predicting the likelihood of altitude sickness or the changes in exercise performance at altitude, are based on the linear relationship between $\dot{V}_E$ and pulse O_2 saturation (S_pO_2) (**Figure 19A**) during steady-state (Lhuissier *et al.*, 2012; Richalet *et al.*, 2012; Bourdillon *et al.*, 2014; Pla *et al.*, 2020) or transient periods (Edelman *et al.*, 1973; Solaiman *et al.*, 2014) of hypoxia. However, the prognostic value of HVR for the risk of altitude sicknesses remains debated, though early findings collected on successful climbers reaching the top of Mt. Everest suggested a link between elevated HVR and greater physical condition and lower symptoms of altitude sickness during the ascent to 8848 m (Schoene *et al.*, 1984).

According to the Fick's Law of Diffusion:

$$\dot{V}_{gas} = \frac{A}{T} \times D \times (P_1 - P_2) \qquad (2)$$

where $\dot{V}_{gas}$ represents the volume of gas per unit of time, A is the surface area available for gas exchange, T is the thickness of the alveolar-capillary membrane, D is the diffusion coefficient of the gas, and ($P_1 – P_2$) is the pressure gradient across the membrane. The primary goal of the HVR is to maintain a functional alveolar-to-arterial O_2 pressure gradient, and thus an effective lung diffusion capacity for O_2 (West & Luks, 2020). Since it is currently impossible to measure A and T In humans, the gas of choice to measure lung diffusive capacity is carbon monoxide due to the high affinity of this gas for hemoglobin (Hb). Existing evidence on the effects of hypoxia on lung diffusive capacity for carbon monoxide (D_LCO) are inconsistent, with increased (Guleria *et al.*, 1971; Dehnert *et al.*, 2010; Agostoni *et al.*, 2011), decreased (Weiskopf & Severinghaus, 1972; Senn *et al.*, 2006), or unchanged (Kreuzer & van Lookeren

Campagne, 1965) D_LCO at different time points withing the first 10 days of exposure to high-altitude. The reason behind these divergent results is not clear (importantly, all these studies had similar ascent rates and relatively similar altitudes), but may relate to individual variability in the ability to recruit and distend the pulmonary vasculature under hypoxic conditions (Steinacker *et al.*, 1998).

While the increased ventilatory drive during acute hypoxia is an essential adaptive mechanism to maintain an efficient O_2 delivery to active tissues, the pulmonary circulation is also affected by hypoxia. Upon exposure (and up to few hours) to hypoxia, increases in pulmonary vascular resistance and pulmonary artery pressure lead to hypoxic pulmonary vasoconstriction (HPV). In conditions of low O_2 availability, this mechanism is critical to match ventilation to perfusion and optimize pulmonary gas exchange by diverting blood flow from poorly ventilated to well ventilated alveoli (Sommer *et al.*, 2016) (**Figure 20**).

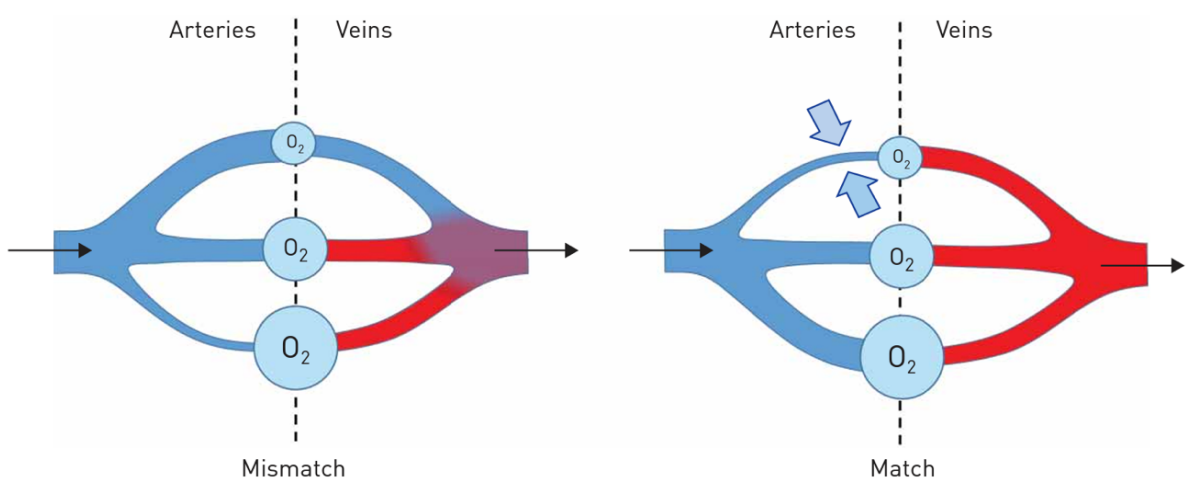

Figure 20. Schematic representation of hypoxic pulmonary vasoconstriction. During acute exposure to hypoxia, pulmonary vasculature constricts and divert deoxygenated blood to alveoli that are better perfused, thereby optimizing ventilation-perfusion matching and improving systemic oxygen delivery. Early animal models demonstrated this vasoconstrictor response to happen at precapillary level withing the pulmonary vascular tree (Kato & Staub, 1966; Parker *et al.*, 1981). Blue arrows indicated the site when constriction of the pulmonary arteries occurs, while the direction of blood flow is shown by black arrows. From Sommer *et al.* (2016).

On the other hand, HPV can become detrimental when activated globally in the lung for a prolonged period of time, as during chronic exposure to hypoxia. In this case, HPV increases pulmonary vascular resistance, thus right heart afterload, and, in the long-term, may stimulate pulmonary vascular remodeling leading to pulmonary hypertension and right heart failure (Naeije & Dedobbeleer, 2013; Williams *et al.*, 2022). This pathological condition is typical of permanent high-altitude residents, although, as highlighted above (*see Chapter 2.1.1*), growing concerns also suggest a link between premature birth, perinatal O_2 therapy and early onset of pulmonary hypertension during young adulthood (Goss *et al.*, 2018a; Goss *et al.*, 2019). It is therefore important to understand whether this later population is susceptible to an increased risk to pulmonary and cardiovascular diseases when moving to high-altitude.

Overall, these changes in pulmonary mechanics, gas exchange, and pulmonary vascular function occurring during acute and chronic hypoxia affects lung function indices assessed by spirometry. In hypobaric hypoxia, the lower air density reduces airway resistance, thus increasing both peak expiratory flow (PEF) and forced expiratory flow between 25 and 75% of total lung volume ($FEF_{25-75\%}$) (Welsh *et al.*, 1993; Cogo *et al.*, 1997; Deboeck *et al.*, 2005), while contradictory results exist regarding both forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) (Welsh *et al.*, 1993; Pollard *et al.*, 1996; Deboeck *et al.*, 2005; Sharma & Brown, 2007). Conversely, normobaric hypoxia, which is commonly achieved by inhaling a nitrogen-enriched gas mixture, have minimal impact on air density (Cross *et al.*, 2018; Netzer *et al.*, 2022) and its effects on lung function in healthy individuals remain underinvestigated.

2.2.2 Cardiovascular system

The cardiovascular system represents an essential link in the O_2 cascade from ambient air to the mitochondria, and therefore it is central during acclimatization to altitude. Both acute and chronic hypoxia induce significant dynamic central (cardiac pump) and peripheral (regional vascular bed) changes within the cardiovascular system that begins upon exposure and evolves over days, weeks, and years of exposure (Bartsch & Gibbs, 2007; Mallet *et al.*, 2021). These cardiovascular changes, which are driven by peripheral chemoreceptor-induced activation of autonomic nervous system (**Figure 16** and **Figure 17**), aim to maintain adequate O_2 delivery in the face of decreased S_aO_2. Keeping in mind *Equation 1*, and that:

$$C_aO_2 = (1.34 \times [Hb] \times S_aO_2) + (0.003 \times P_aO_2) \qquad (3)$$

where, C_aO_2 represents the arterial O_2 content, 1.36 the affinity for O_2 to Hb, and 0.003 the solubility of O_2 dissolved in blood, changes in stroke volume and heart rate (and thus in $\dot{Q}$), and lately in Hb concentration, are critical mechanisms to contrast the drop in S_aO_2.

2.2.2.1 Cardiac function and macrocirculation

In addition to stimulate the rise in $\dot{V}_E$ (**Figure 16** and **Figure 17**), hypoxia-induced increases in peripheral chemoreceptors discharge stimulate cardiac sympathetic activity to increase heart rate and myocardial contractility (Marshall, 1994). The increase in heart rate results from a combination of increased sympathetic activity and parasympathetic withdrawal (Koller *et al.*, 1988), and represents a primary mechanisms by which O_2 delivery is maintained during acute hypoxia (Vogel & Harris, 1967). On the contrary, during the first hours of exposure, stroke volume remains at sea-level values, due to similar reductions in LV end-diastolic and end-systolic volumes (Baggish *et al.*, 2014). However, stroke volume begins to decrease over the first few days at elevations higher than 3000 m due a greater drop in end-diastolic vs. end-

systolic volume, leading to a $\dot{Q}$ comparable to sea-level (Klausen, 1966). A brief description of the change in heart rate, stroke volume, Q over the first few days of exposure at high-altitude is shown in **Figure 21**.

Alongside with changes in LV hemodynamics, the onset of hypoxic pulmonary vasoconstriction within the first 5 minutes of exposure leads to increases in pulmonary artery pressure (Motley *et al.*, 1947), and thus in RV internal diameter (**Figure 22**) (Netzer *et al.*, 2017). In contrast, LV diameter seems to slightly decrease during acute hypoxic exposure. The mild reduction in LV chamber dimension results from direct interaction with the RV via compression of the interventricular septum, rather than increases in LV afterload (**Figure 22**) (Williams *et al.*, 2022). Indeed, LV afterload is unaffected by acute hypoxia since the sympathetically-mediated vasoconstriction is balanced by hypoxia-induced peripheral vasodilation (Simpson *et al.*, 2021).

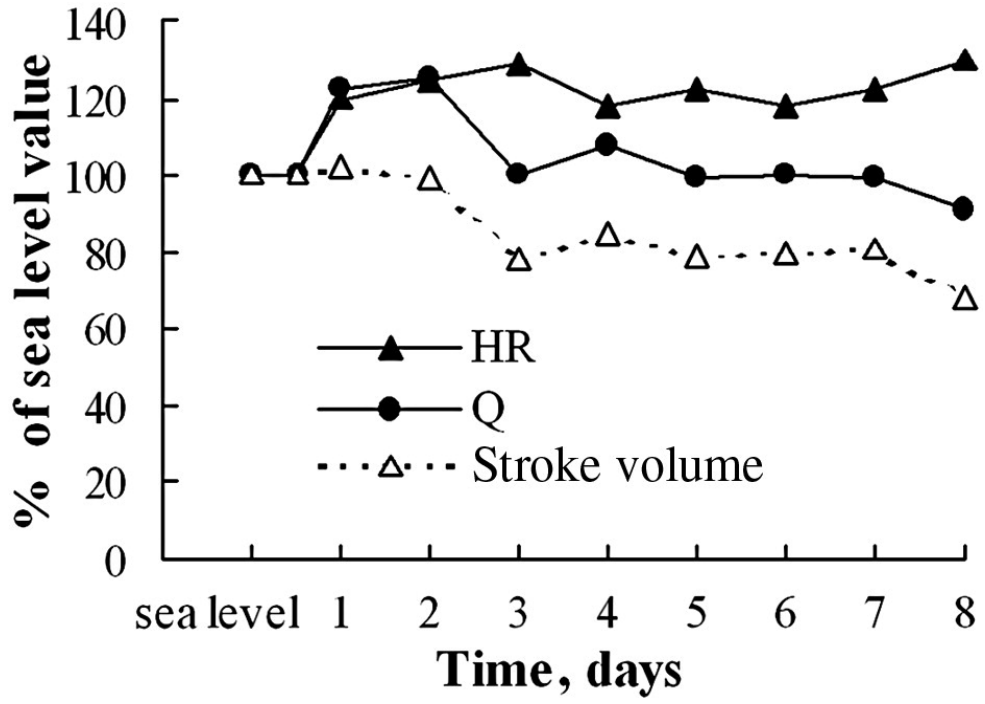

Figure 21. Mean heart rate (HR), cardiac output (Q), and stroke volume changes during the first days of exposure to hypoxia (3800 m) in healthy volunteers. Both Q and HR increase within the first 24 hours after arrival at high-altitude, while SV decreases below normoxic values after 2 days of exposure. After 8 days of high-altitude sojourn, Q return sea-level values, while both HR and SV remain higher and lower, respectively, compared to sea-level. From Naeije (2010).

Despite these pressure and structural changes within the heart, global cardiac function is preserved in acute hypoxia, as demonstrated by similar ejection fractions and filling pressures in both the RV and LV using echocardiography (Huez *et al.*, 2005). Recent findings from research expeditions to high-altitude confirmed subtle, though significant, increases in regional cardiac mechanics in lowlanders upon arrival at altitude. LV twist and both LV and RV longitudinal and systolic strains increase, despite small reductions in stroke volume, in lowlanders acutely exposed to hypoxia (Huez *et al.*, 2005; Stembridge *et al.*, 2014; Stembridge *et al.*, 2015; Williams *et al.*, 2019). A summary of these mechanisms is shown in **Figure 23**.

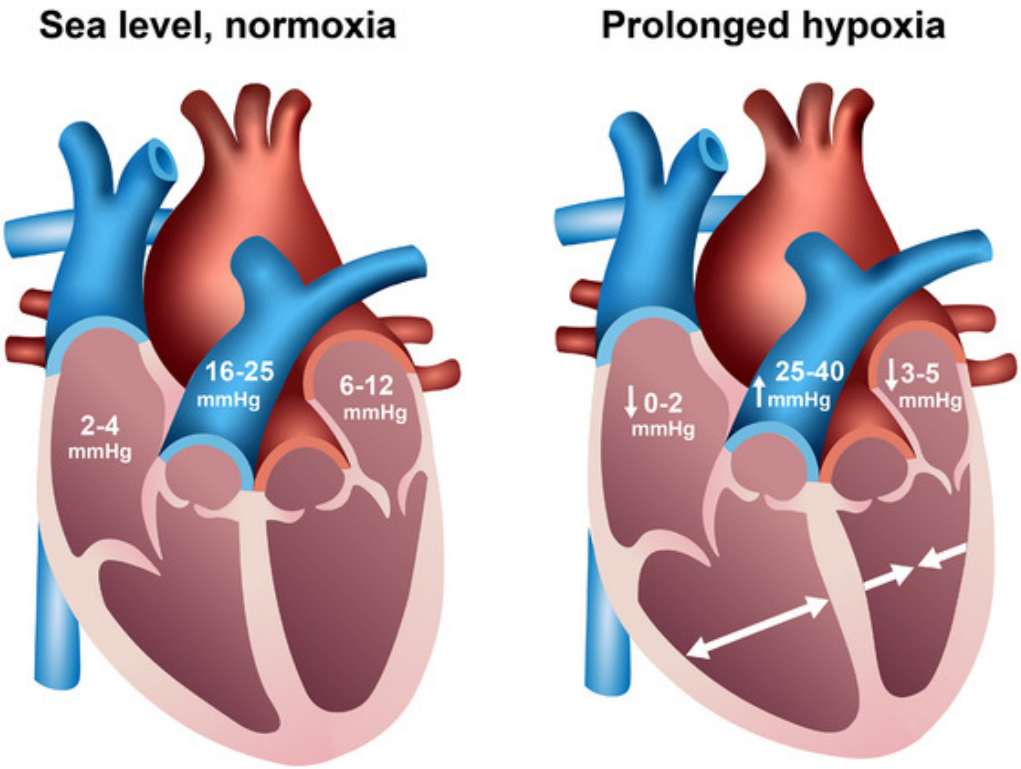

Figure 22. Changes in cardiac chambers dimensions and pressures over few days of exposure to hypoxia. Compared to normoxia (left picture), hypoxia lowers both right and left atrial pressure (reduced filling due to lower blood volume), while it increases pulmonary artery pressure, and thus imposes a greater afterload to the right ventricle. In turn, right ventricle cardiac output is lowered and the chamber diameter increased. This latter mechanism, via greater interventricular pressure, decreases left ventricle internal dimensions with no changes in LV afterload (balanced by local peripheral vasodilation). From Williams *et al.* (2022).

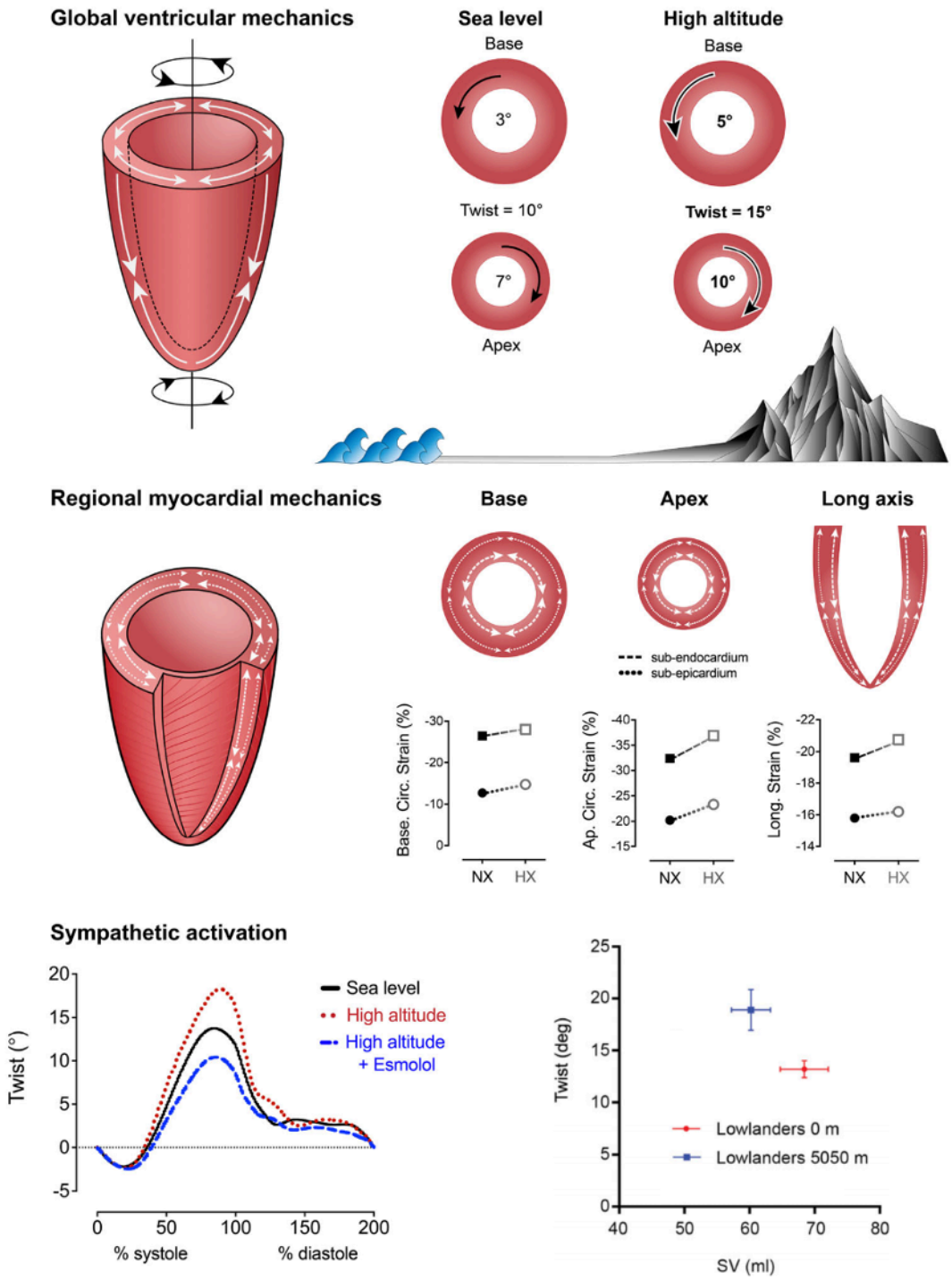

Figure 23. Graphical representation and experimental data of the adjustments in cardiac left ventricular (LV) mechanics elicited by acute hypoxia at rest. In the upper panel, acute and chronic hypoxia elevate global LV mechanics as a result of increased LV twist (rotation) at both the base and apex. At regional level (intermediate panel), subendocardial (square symbols, dashed line) and subepicardial mechanics (open circle symbols, dotted line) seem to be maintained in hypoxia compared to normoxia, as demonstrated by consistent increases in local strain in the basal (left), apical (middle) and longitudinal axes. Lastly (bottom panels), greater sympathetic activation during acute hypoxia is largely responsible for augmented LV twist mechanics during systole. Of note, the increase in LV twist in hypoxia is an important adaptive response to lower resting stroke volume at altitude. *HX*, hypoxia; *NX*, normoxia; *SV*, stroke volume. Modified from Stembridge *et al.* (2014) and Williams *et al.* (2022).

2.2.2.2 Peripheral microcirculation

At peripheral level, acute hypoxia elevates skeletal muscle blood flow (a phenomenon termed hyperemia) proportionally to the decrease in C_aO_2 (Gonzalez-Alonso *et al.*, 2006) via local vasodilation and decreased systemic vascular resistance (Rowell & Blackmon, 1989; Wilkins *et al.*, 2006). However, this vascular response contradicts hypoxia-induced activation of the sympathetic nervous system, which is known to stimulate a global vasoconstriction response (Saito *et al.*, 1988; Halliwill & Minson, 2002). With exception of pulmonary vasculature where hypoxia elicits an important vasoconstrictor response, the sensitivity of the skeletal muscle vasculature to sympathetic vasoconstriction is reduced during acute systemic hypoxia (Dinenno, 2016). At this regard, functional sympatholysis has been shown to be of paramount importance for the microcirculatory response to hypoxia (**Figure 24**) (Casey & Joyner, 2012).

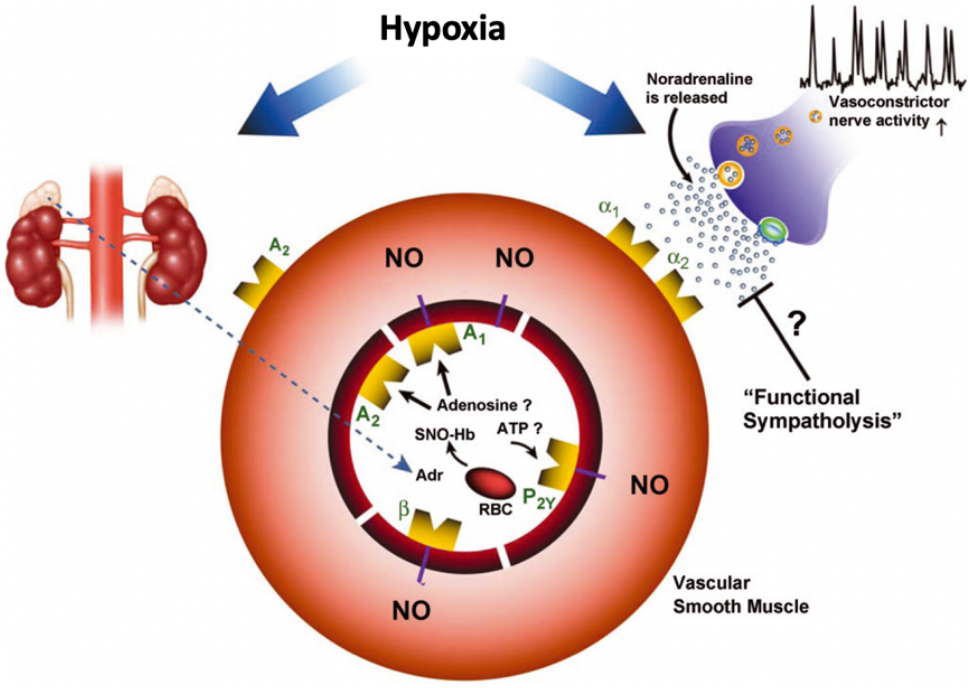

Figure 24. Proposed mechanisms for hypoxia-induced vasodilatation at rest and during exercise. Hypoxia activates the sympathetic nervous system, which stimulates systemic vasoconstriction. However, this sympathetically-mediated vasoconstrictor response is counterbalanced by hypoxia-induced vasodilation (aiming maintain adequate oxygen delivery to peripheral tissues). The reduced vasoconstrictor response to sympathetic activation is termed functional sympatholysis. α_1 and α_2 indicate α_1 and α_2 adrenergic receptors, respectively; A_1 and A_2, adenosine receptors; β and β_2 adrenergic receptors; *Adr*, adrenaline. From Casey and Joyner (2012).

Importantly, skeletal muscle vasodilation in hypoxia is not mediated by the autonomic nervous system, as demonstrated by lack of vasodilatory response to hypoxia following forearm infusion of beta-adrenergic blockade (Richardson *et al.*, 1967). Instead, the metabolic activity of the red blood cells, and especially their greater release of adenosine triphosphate in hypoxia, is likely the main factor driving hypoxia-induced vasodilation (Gonzalez-Alonso *et al.*, 2006). Furthermore, NO also represents a critical signaling molecule regulating global and regional distribution of blood flow. The potential role of NO in modulating hypoxic vasodilation was first described in rabbits (Pohl & Busse, 1989) and later confirmed by a two-fold increase and a ten-fold greater circulating NO metabolites in young lowlanders (Levett *et al.*, 2011; Rasica *et al.*, 2021b) upon arrival at altitude and in high-altitude residents (Erzurum *et al.*, 2007), respectively. However, the key stimulator of NO production and release is blood flow *per se*, via increased non-linear shear stress generated over endothelial surface of the vascular walls (Balligand *et al.*, 2009).

Ultimately, the balance between sympathetic vasoconstriction and hypoxic vasodilation during acute hypoxic exposure determines blood pressure. Upon arrival at altitude, hypoxic vasodilation prevails over sympathetic vasoconstriction, as shown by mild decreases in peripheral vascular resistance and thus in blood pressure (Vogel & Harris, 1967; Levine *et al.*, 1997). However, after few hours/days of exposure, the balance shifts towards predominant vasoconstriction, sometimes leading to mild hypertension (Wolfel *et al.*, 1994).

2.2.3 Cerebrovascular system

The brain is the most O_2-dependent organ in the human body, accounting for ~20% of the resting total body O_2 consumption under normal physiological conditions (Jain *et al.*, 2010). This inordinate oxidative metabolism makes the brain particularly vulnerable in hypoxic condition. Remarkably, various adaptive mechanisms both at systemic level and within the

cerebrovasculature (**Figure 25**) instantly respond to hypoxia aiming to maintain adequate O_2 supply to the brain.

These mechanisms largely depend on decreases in P_aO_2 and involved a complex interaction of several physiological, metabolic, and biochemical processes.

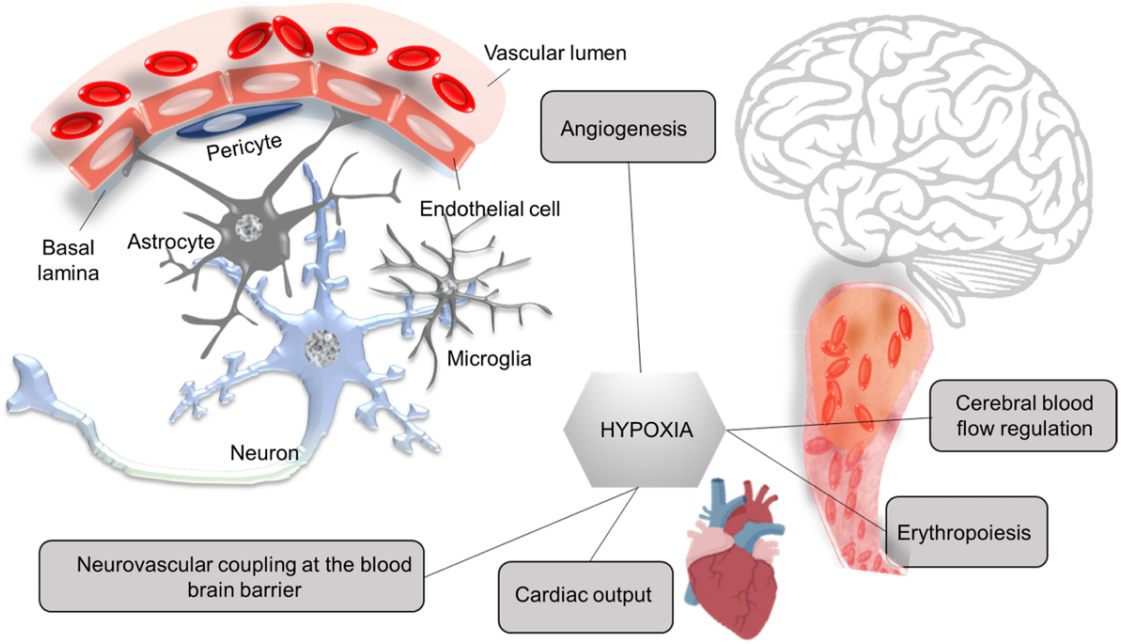

Figure 25. Hypoxia elicits several cardiovascular responses and adaptations which improve cerebral blood flow and cerebrovascular function, thus enhancing cerebral oxygenation. From Mallet *et al.* (2021).

Equation 3 can be rearranged to calculate cerebral O_2 delivery (cO_2delivery) as:

$$cO_2delivery = CBF \times C_aO_2 \qquad (4)$$

Thus, cerebral homeostasis in hypoxic conditions hinges upon an increase in CBF, primarily through hypoxic cerebral vasodilation, and a mitigation of hypoxemia, primarily through hyperventilation (*see Chapter 2.2.1*). Consistently, the increase in CBF with severe hypoxemia (when P_aO_2 drops below 50 mmHg) is immediate within few minutes upon exposure (Severinghaus *et al.*, 1966; Huang *et al.*, 1987) and it remains elevated for a few days in hypoxia (**Figure 26A**) (Lucas *et al.*, 2011; Subudhi *et al.*, 2014; Willie *et al.*, 2014a). The relationship between CBF augmentation and altitude is exponential (**Figure 26B**), with a linear

trend up to ~6000 m followed by an abrupt increase in CBF due to marked vasodilation of the cerebral arteries up to 8848 m (Mt. Everest). These changes in cerebral hemodynamics result in a modest increase in cO_2delivery, which return to sea-level values after a few days of exposure (**Figure 26A**).

The factors regulating the rise in CBF in response to decreases in C_aO_2 are multiple (i.e., MAP, hematology, free radicals), yet can be summarized as the relative strength of four primary mechanisms (**Figure 27**): *a*) hypoxic ventilatory response (respiratory alkalosis); *b*) hypercapnic ventilatory response; *c*) hypocapnic cerebral vasodilation, and *d*) hypocapnic cerebral vasoconstriction (Ainslie & Subudhi, 2014).

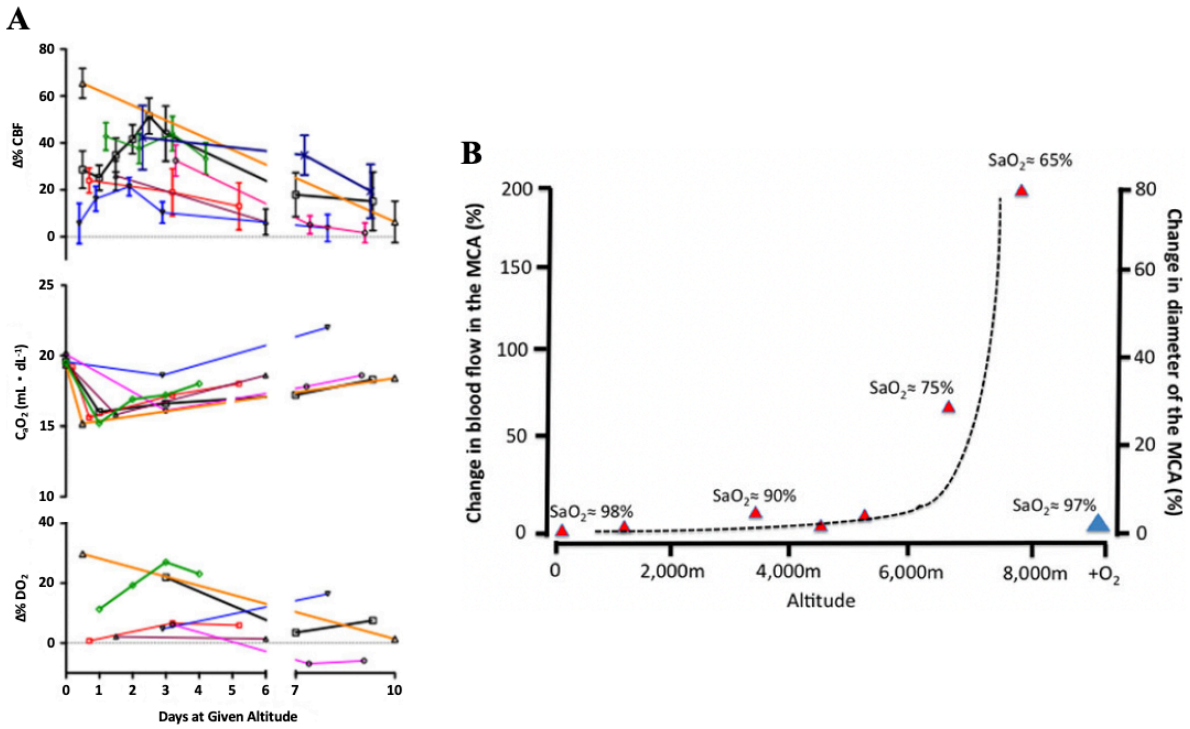

Figure 26. Percent increases in cerebral blood flow (Δ%CBF) in response to absolute decreases in arterial oxygen content (C_aO_2) with time at high-altitude collected from six studies at various altitudes (ranging from 3475 m to 5050 m) (Panel A). Changes in cerebral oxygen delivery (Δ%DO_2) over time at altitude are also shown (Panel A). In Panel B, percent changes in CBF in the middle cerebral artery (MCA) and MCA diameter with increasing altitude (up to 7950 m). The blue triangle shows a sudden drop in CBF back to sea-level values upon breathing supplemental oxygen (+O_2). Modified from Ainslie and Subudhi (2014).

The increase in CBF due to cerebral vasodilation (at arteriolar level, and in major arteries in severe hypoxia – **Figure 26B**) represents the first cerebrovascular response to decreases in P_aO_2. However, this increased hemodynamic drive is rapidly depressed by the drop in P_aCO_2, consequence of the HVR, which promotes cerebral vasoconstriction (Brugniaux *et al.*, 2007). Indeed, the cerebrovasculature is extremely sensitive to fluctuations in P_aCO_2 rather than in P_aO_2, though reduced P_aO_2 becomes the predominant driver to augment CBF for P_aO_2 below ~45 mmHg (corresponding to ~5000 m) (**Figure 28**).

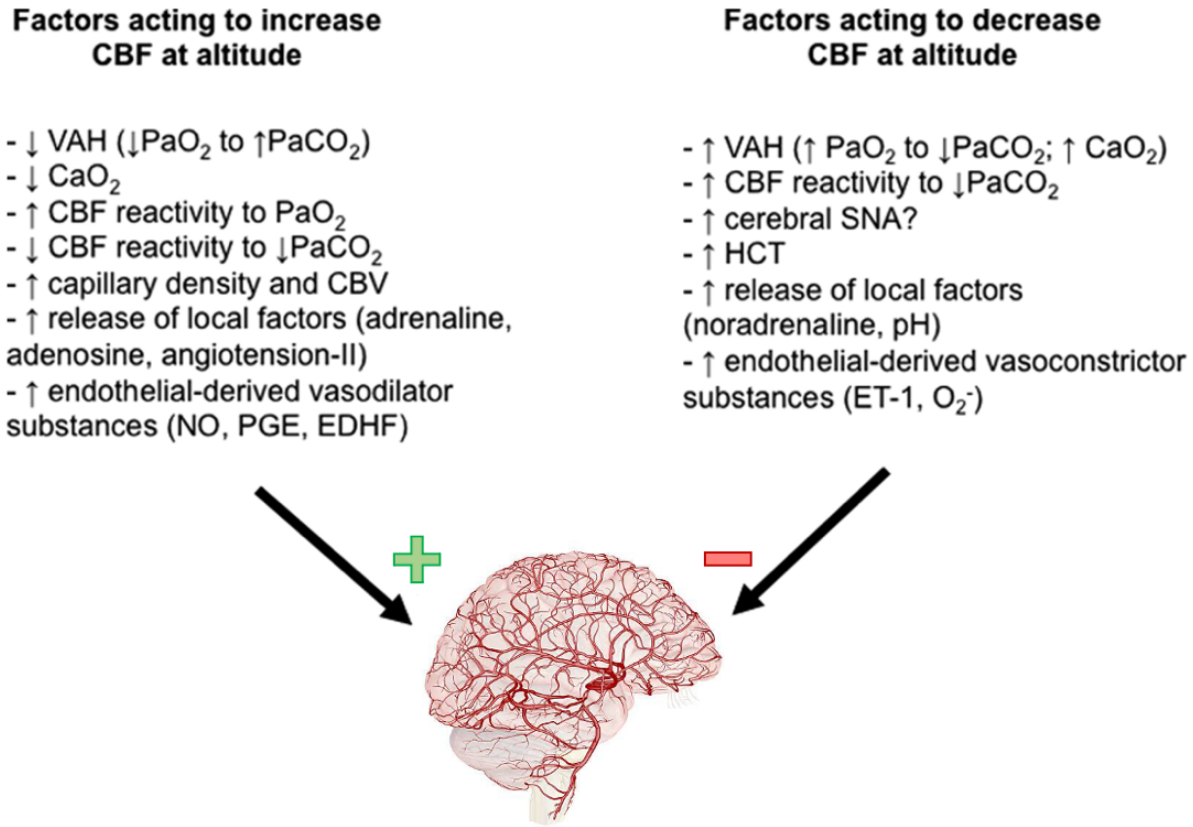

Figure 27. Summary of the primary factors and mechanisms increasing or decreasing cerebral blood flow (CBF) during hypoxic exposure. *VAH*, ventilatory acclimatization to hypoxia; *PaO_2*, arterial partial pressure of oxygen; *$PaCO_2$*, arterial carbon dioxide partial pressure; *CBV*, cerebral blood volume; *NO*, nitric oxide; *PGE*, prostaglandins; *EDHF*, endothelium-derived hyperpolarizing factor; *SNA*, sympathetic nervous activity; *HCT*, hematocrit; *ET-1*, endothelin-1; *O_2^-*, superoxide. Modified from Ainslie and Subudhi (2014).

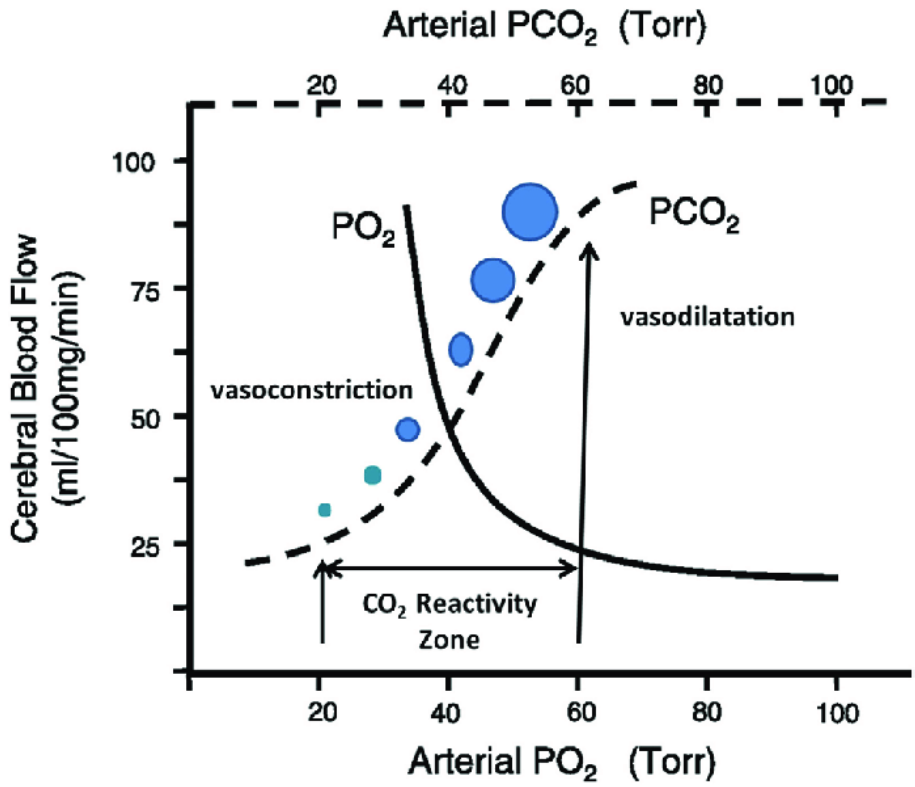

Figure 28. Schematic representation of the competing influence of arterial partial pressure of oxygen (PO_2) and carbon dioxide (PCO_2) on cerebral blood flow regulation. Upon exposure to hypoxia, the fall in arterial PO_2 causes vasodilation, especially when it drops below ~45 mmHg. However, the reduction in arterial PO_2 also triggers an hyperventilatory reflex response via direct peripheral chemoreceptors stimulation. In turn, the hyperventilation-induced decrease in arterial PCO_2 blunts the vasodilatory response by causing cerebral vasoconstriction. The balance between arterial levels of PO_2 and PCO_2 (PO_2/ PCO_2 ratio), mediated by changes in ventilatory control, is the main determinant of CBF in hypoxic conditions. Of note, when arterial PO_2 falls below ~45 mmHg, the vasodilatory stimulus likely predominates over the decrease in arterial PCO_2. From Godoy et al. (2017).

Therefore, the overall CBF response during acute and chronic hypoxia is regulated by the balance in arterial blood gas (P_aO_2/P_aCO_2) (Lucas *et al.*, 2011) – hence on cerebrovascular reactivity to O_2 and CO_2 - and, although to a lesser extent, by the degree of change in MAP (*see Chapter 2.2.2*) leading to a loss of cerebral autoregulation (Levine *et al.*, 1999).

Previous studies have produced variable results, with either increased (Jensen *et al.*, 1990; Ainslie *et al.*, 2012; Fan *et al.*, 2015; Fluck *et al.*, 2015; Aebi *et al.*, 2020), unchanged (Willie *et al.*, 2015), decreased (Ainslie *et al.*, 2007; Rupp *et al.*, 2014), or selectively altered (Lucas *et al.*, 2011) cerebrovascular CO_2 reactivity in hypoxic conditions compared to normoxia. The discrepancy is likely due to different degrees of hypoxia and exposure length between studies,

with an unclear role played by adaptation or maladaptation (i.e., AMS, orthostatic intolerance) to hypoxia (Jensen *et al.*, 1990; Jansen *et al.*, 1999; Blaber *et al.*, 2003).

Although small in magnitude, the hypoxia-induced change in BP may affect CBF, thus cerebral autoregulation. Although the mechanisms governing cerebral autoregulation are many and still unclear, a consistent body of research demonstrated a loss of cerebral autoregulation upon exposure to hypoxia, which persists over days, months, and years of permanence at altitude (Levine *et al.*, 1999; Ainslie *et al.*, 2007; Jansen *et al.*, 2007; Bailey *et al.*, 2009; Iwasaki *et al.*, 2011). It seems therefore that an intact cerebral autoregulatory response to pressor changes is not a hallmark mechanism of normal adaptation to hypoxia.

2.2.4 Skeletal muscle

Skeletal muscle is the final step in the O_2 cascade and exposure to hypoxia significantly affects O_2 diffusion (via reduced O_2 pressure gradient) within the skeletal muscle microcirculation (**Figure 14**) (West & Luks, 2020). Thanks to its remarkable plasticity, significant changes occur in skeletal muscle structure and function, including changes in fiber type composition, capillarization, and mitochondrial content, with major differences between acute and chronic (several week) or lifelong exposure to hypoxia (Hoppeler, 2016). Ultimately, these responses aim to increase O_2 diffusion and utilization at skeletal muscle level, hence to preserve physical work capacity under conditions of low O_2 availability. For the scope of this thesis we will briefly discuss only changes in skeletal muscle mitochondrial and microvascular function occurring in acute hypoxia [for chronic adaptations refer to reviews: Murray and Horscroft (2016), Favier *et al.* (2015), and Horscroft and Murray (2014)].

At skeletal muscle and mitochondrial levels, among other mechanisms, Hypoxia Inducible Factor 1, and especially its subunit α (HIF-1α), is a primary mechanism involved in the acute response to low O_2 availability (**Figure 29**) (Semenza, 2001).

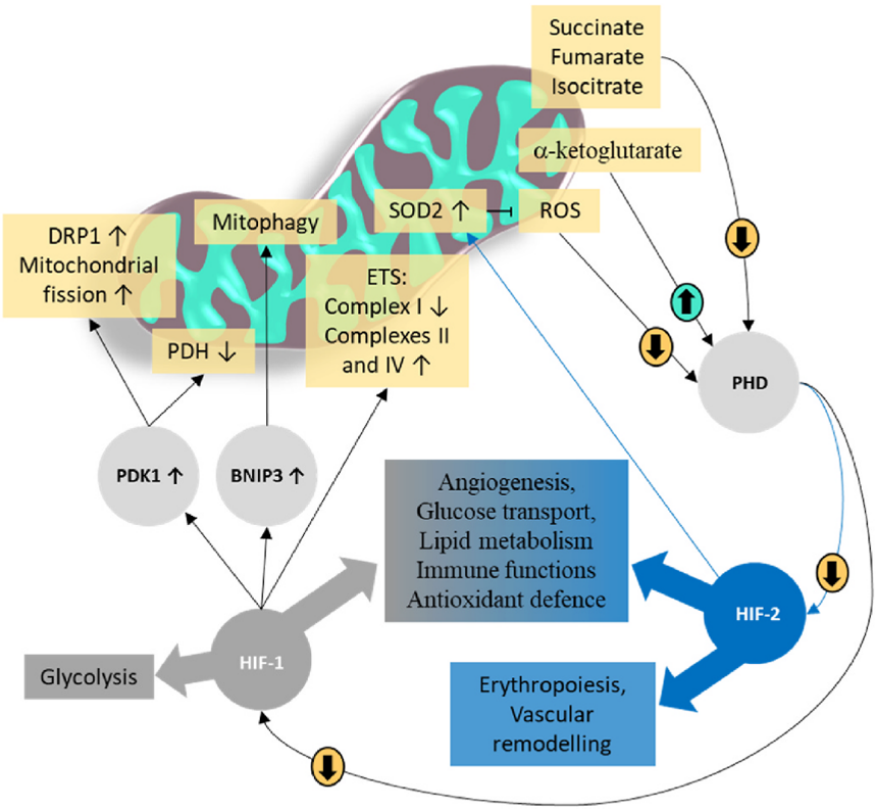

Figure 29. Interactions between hypoxia inducible factor (HIF) and mitochondria. *PHD*, prolyl hydroxylases; *ROS*, reactive oxygen species; *SOD2*, mitochondrial superoxide dismutase; *Complex I – Complex IV*, complexes of the respiratory system; *DRP1*, dynamin-related protein 1; *PDK1*, pyruvate dehydrogenase kinase 1; *PDH*, pyruvate dehydrogenase; *BNIP3*, BCL2 Interacting Protein 3. From Burtscher *et al.* (2023).

In cell cultures, the activation of the HIF-1α pathway began immediately upon reduction in the O_2 concentration from normoxic levels (20.93%), though the greater magnitude of the response was observed for cellular O_2 concentrations below 6% (Jiang *et al.*, 1996). Furthermore, exposure to hypoxia also increases the expression of another signaling molecule, the proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) (Murray, 2009). Both HIF-1α and PGC-1α begin transcriptional processes within the skeletal muscle aiming to increase O_2 extraction capacity and O_2 utilization (Lemieux & Birot, 2021). In particular, while HIF-1α is primarily responsible for the angiogenic (via increases in Vascular Endothelial Growth Factor, VEGF) and erythropoietic response to hypoxia (Semenza, 2001), PGC-1α mediates both angiogenic mechanisms (by greater VEGF expression) and mitochondrial biogenesis (**Figure 30**) (Murray, 2009).

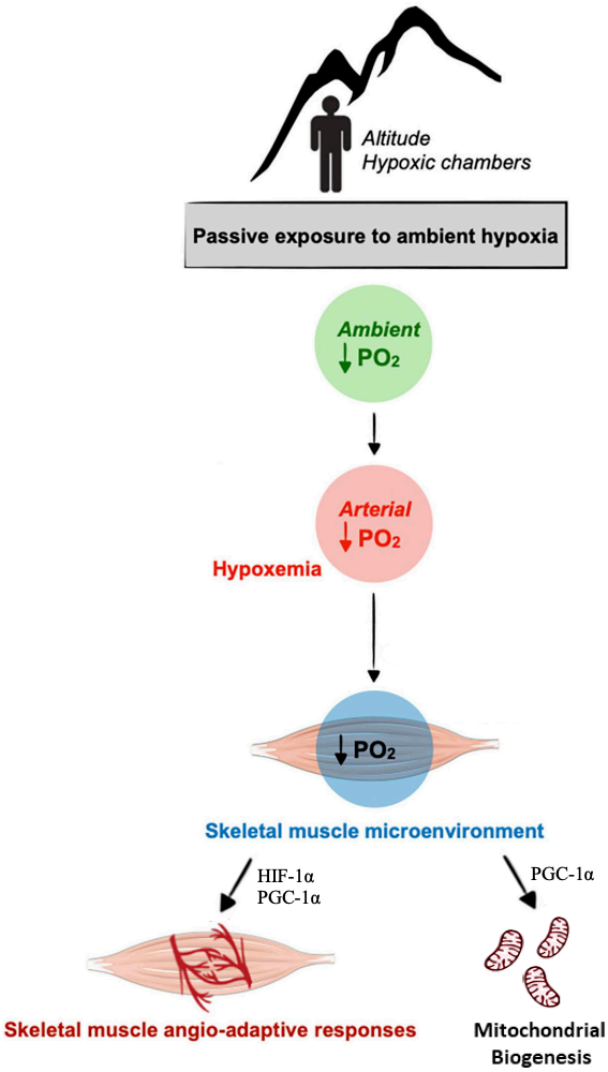

Figure 30. Schematic representation of the effects of low oxygen availability (i.e., hypoxia) on skeletal muscle. A reduction in ambient partial pressure of oxygen (PO_2) induces a consequent drop in arterial and capillary PO_2. Within the skeletal muscle, the low PO_2, via activation of both the HIF-1α and PGC-1α pathways, stimulates the formation of new micro vessels (angiogenic response) and mitochondria. Like in other systems, the aim of these response is to maintain an adequate O_2 delivery to sustain physical work through oxidative metabolic pathways. Modified from Lemieux and Birot (2021) and Li *et al.* (2020).

2.2.5 Oxidative stress and nitric oxide pathway

The activation of several cellular pathways (including HIF-1α and PGC-1α) by exposure to hypoxia may also produce an imbalance between oxidative stress and antioxidant capacity. This has been demonstrated by a consistent body of literature, as rapid increases in plasma levels of oxidative stress biomarkers were found within 24 h of exposure to both normobaric

and hypobaric hypoxia (Ribon *et al.*, 2016; Strapazzon *et al.*, 2016; Debevec *et al.*, 2017; Martin *et al.*, 2020), though greater ROS are reported in hypobaric compared to normobaric hypoxic conditions (Faiss *et al.*, 2013; Ribon *et al.*, 2016). The factors behind ROS overproduction in hypoxia are many and involve a complex interplay of several cellular and molecular pathways (which also likely differ between tissues and organs), yet it seems to be related to reductive stress within the mitochondria (Duranteau *et al.*, 1998), augmented catecholamine production (Mazzeo *et al.*, 1998), decreased mitochondria redox potential (Kehrer & Lund, 1994), and lower activation of the xanthine oxidase (XO) pathway (Yuan *et al.*, 2004). In turn, this exaggerated response might dampen the antioxidant system, limit NO bioavailability (Faiss *et al.*, 2013), as well as modulate the HVR (Pialoux *et al.*, 2009a; Pialoux *et al.*, 2009b; Raberin *et al.*, 2021). Furthermore, rodent studies showed organ damage, especially in the brain and the heart, induced by the greater oxidative stress triggered by acute exposure to hypoxia (Jing *et al.*, 2021). This is particularly relevant in the present research project since, as reviewed in the previous sections (*see Chapter 2.1.2*), prematurely born adults presents a unique cardiovascular phenotype which may predispose them to an increased risk to develop cardiovascular disease.

Ultimately, ROS overproduction in hypoxia, if not compensated by a balanced rise in antioxidant activity, may, either directly or indirectly, contribute to the onset and rapid progression of high-altitude disorders (i.e., AMS, high-altitude pulmonary edema, chronic mountain sickness) (Pena *et al.*, 2022).

A further central signaling molecule involved in the acute and chronic response to hypoxia is NO. Cumulative evidence suggests that NO synthesis and metabolites are increased upon exposure to hypoxia and remain constantly higher during acclimatization (Rasica *et al.*, 2021b). The NO time-course in hypoxia is further supported by studies conducted in native highlanders who showed persistent increased serum, urinary, and salivary levels of NO metabolites

(Erzurum *et al.*, 2007). Consequently, elevated NO levels lead to higher blood flow and thus greater O_2 delivery to offset hypoxia. Indeed, hypoxia-induced vasodilation both at peripheral (with except of pulmonary vasculature) and cerebral level (*see Chapters 2.2.2 and 2.2.3*), which is in part mediated by NO (Casey *et al.*, 2010; Beall *et al.*, 2012; Umbrello *et al.*, 2013), is well described in the literature (Casey *et al.*, 2014; Dinenno, 2016). At this regard, insufficient augmentation of NO synthesis at altitude was associated with failure to acclimatize. Exaggerated hypoxic pulmonary vasoconstriction response leading to early onset of high-altitude pulmonary edema (HAPE) was observed in adults with reduced pulmonary NO bioavailability during exposure to at 4559 m (Bailey *et al.*, 2010).

Therefore, increases in NO production and bioavailability represent key responses to acute hypoxia driving an important vasodilatory response in the peripheral and cerebral circulations, as well as modulating the magnitude of hypoxic pulmonary vasoconstriction (**Figure 24**). As previously reviewed in this thesis, these are central mechanisms behind a successful acute response and acclimatization to hypoxia.

2.2.6 Exercise capacity

A decline in exercise capacity is a universal experience among all who are exposed to either terrestrial or simulated altitude, with the magnitude of the decrease being dependent on the degree and duration of hypoxia, as well as the type and intensity of the exercise performed. In general, during acute hypoxic exposure, $\dot{V}O_{2max}$ begins to decrease from low-altitude and upwards, and it is estimated a ~1% reduction for every 100 m of altitude gained (**Figure 31A**) (Fulco *et al.*, 1998), although a high interindividual variability in the magnitude of this response has been observed (MacInnis *et al.*, 2015). Furthermore, the drop in $\dot{V}O_{2max}$ al altitude seems to be more pronounced (~1%/1000 m of elevation) in trained, compared to untrained, age-matched individuals (**Figure 31B**) (Fulco *et al.*, 1998; Wehrlin & Hallen, 2006; MacInnis *et*

al., 2015), likely due to greater arterial O_2 desaturation (Gore *et al.*, 1996) secondary to higher alveolar-end-capillary O_2 diffusion limitation and ventilation-perfusion mismatch (Wagner *et al.*, 1987).

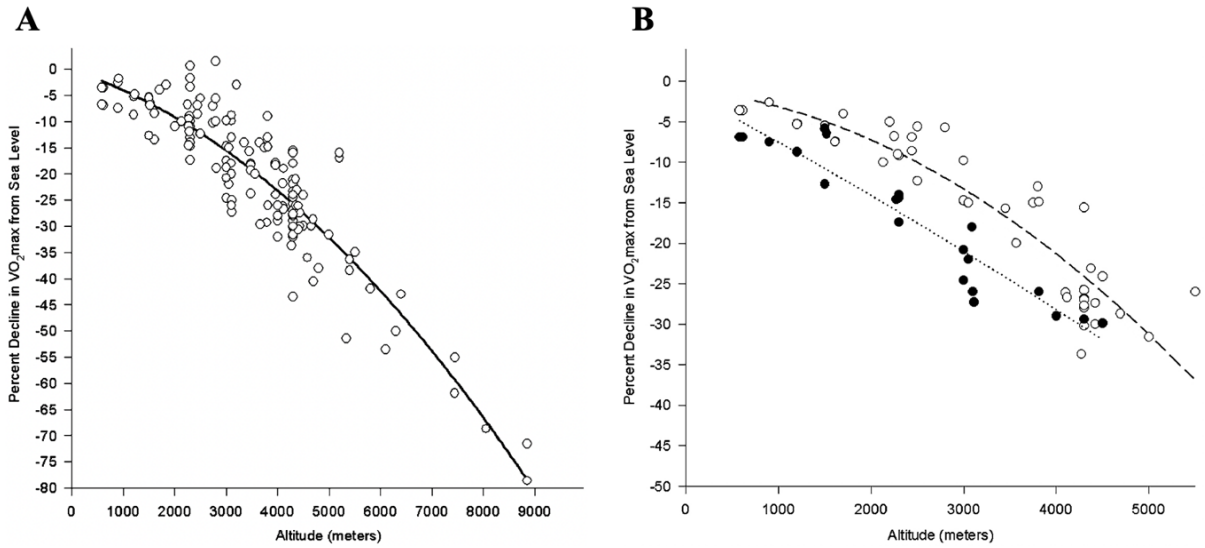

Figure 31. Decrease in maximal oxygen consumption ($\dot{V}O_{2max}$) with increasing altitude (panel A). Each point represents the average $\dot{V}O_{2max}$ decrease in individuals tested at actual or simulated altitude ranging from 580 m to 8848 m in a total of 67 studies. Therefore, the regression line drawn in panel A include thousands of measured $\dot{V}O_{2max}$ values. In panel B, decrease in $\dot{V}O_{2max}$ with increasing altitude in trained (black circles – dotted line) and untrained (white circles – broken line) individuals. Of note, the relationship is linear in trained individuals and curvilinear in untrained participants. Modified from Fulco *et al.* (1998).

$\dot{V}O_{2max}$ is a physiological characteristic determined by the integration of the parametric limits of both the Fick equation (rearranged from *Equation 1*) and the Fick's Law of Diffusion:

$$\dot{V}O_2 = SV \times HR \times (C_aO_2 - C_vO_2) \quad (1)$$

$$\dot{V}O_2 = DO_2 \times (P_{cap}O_2 - P_{mito}O_2) \quad (5)$$

where, SV represents stroke volume, HR heart rate, C_vO_2 the venous O_2 content, DO_2 the O_2 diffusing capacity, $P_{cap}O_2$ and $P_{mito}O_2$ the partial pressure of O_2 at capillary and mitochondrial level, respectively. More specifically, in *Equation 1*, stroke volume can be rewritten as the difference between LV end-diastolic volume and end-systolic volume. *Equation 1* described the convective factors of O_2 transport from ambient air to mitochondria, while *Equation 2* the

diffusive (both at pulmonary and skeletal muscle level) mechanisms involved in the O_2 cascade (Wagner, 1988, 1991, 1993).

Considering the physiological pulmonary, cardiovascular, and skeletal muscle responses and adaptations elicited by acute hypoxic exposure reviewed in the previous sections, it is clear that hypoxia affects both convective and diffusive mechanisms involved in the O_2 transport and that determine $\dot{V}O_{2max}$. In particular, at peak exercise intensity lung O_2 diffusion capacity (via reduced O_2 gradient across alveolar-end-capillary membrane) and O_2 transport in the blood (via decreased S_aO_2 and dissolved O_2 in blood, hence lower C_aO_2) are decreased in conditions of hypoxia (Reeves *et al.*, 1987; Wagner *et al.*, 1987). Surprisingly, a recent impressive work conducted in acute severe normobaric hypoxia demonstrated for the first time the presence of a large functional reserve of skeletal muscle O_2 diffusion capacity (DmO_2), which is recruited during maximal and supramaximal exercise in hypoxia (Calbet *et al.*, 2015). Therefore, as shown in **Figure 32**, exercise capacity in hypoxic condition is primary impaired by O_2 delivery

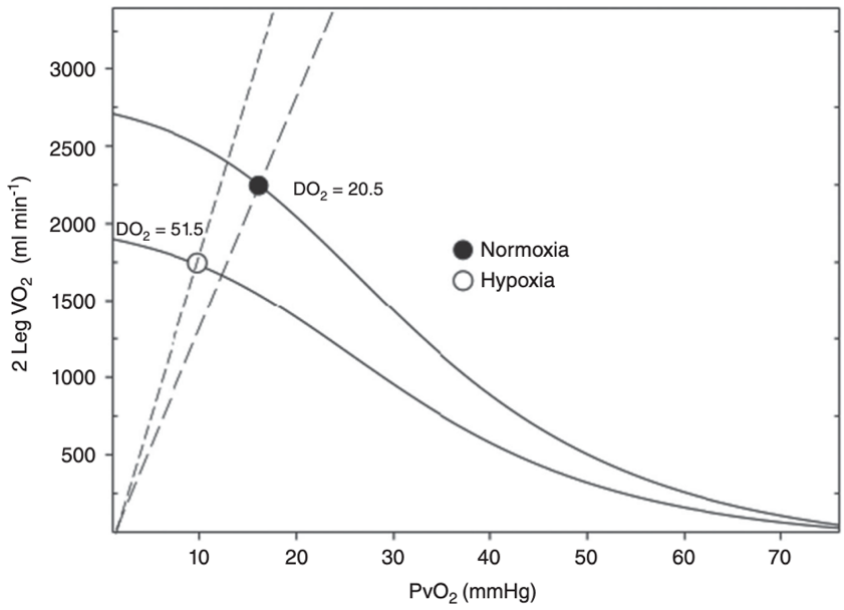

Figure 32. Wagner diagram showing convective (Fick Principle, *Equation 1*, solid line) versus diffusive (Fick's Law of Diffusion, *Equation 2*, dotted line) limitations during maximal exercise in normoxia and acute severe normobaric hypoxia (P_IO_2 = 73 mmHg). The intersection point (DO_2) of these two lines represents the maximum oxygen consumption ($\dot{V}O_{2max}$). Despite lower O_2 delivery in hypoxia (represented by the vertical displacement of DO_2), the steeper slope of the broken line in hypoxia shows a higher skeletal muscle O_2 diffusion compared to

normoxia (horizontal displacement of DO_2). Importantly, in this study, leg $\dot{V}O$ at maximal intensity was calculated using the Fick Principle from direct measures of leg blood flow (by thermodilution technique) and arterio-venous O_2 difference across the muscle (by catheters), and therefore lung O_2 diffusion was not taken into account. In reality, O_2 diffusion across the alveolar-end-capillary membrane is reduced by hypoxia (Reeves *et al.*, 1987; Wagner *et al.*, 1987), resulting in similar overall diffusive capacity (sum of skeletal muscle and lung O_2 diffusion) at peak exercise intensity in hypoxia compared to normoxia (Wagner, 2011). Modified from Calbet *et al.* (2015).

rather than diffusion constraints at skeletal muscle level. Moreover, some mechanisms within the mitochondria might also be involved in lowering exercise performance in hypoxia (Calbet *et al.*, 2015). Indeed, despite the activation of Hypoxia Inducible Factors and PGC-1α pathways by both exercise and hypoxia, the interplay of mitochondria and these pathways during hypoxic exercise is not well understood yet.

2.3 Premature birth and exposure to hypoxia

As reviewed in *Chapter 2.1*, premature birth confers long-term effects on several organs and functions within the body, especially in the respiratory, cardiopulmonary, and cardiovascular systems. As extensively explained in *Chapter 2.2*, an efficient acute response to hypoxia relies on these systems, and successful adaptation to hypoxia might therefore be compromised in adults born prematurely leading to an increased risk for altitude sickness and/or cardiovascular event during hypoxic exposure (i.e., sojourn at altitude). However, the scientific community has only recently started to investigate the combined effects of premature birth and hypoxia, either at rest or during exercise, in preterm individuals during adulthood.

Although still limited, animal models and human studies suggest specific ventilatory, cardiopulmonary, and cardiovascular responses to hypoxia in preterm born individuals both at rest (Rehan *et al.*, 1996; Nock *et al.*, 2004; Bates *et al.*, 2014; Agrawal *et al.*, 2020; Barton *et al.*, 2021) and during exercise (Farrell *et al.*, 2015; Laurie *et al.*, 2018; Debevec *et al.*, 2019; Haraldsdottir *et al.*, 2019; Barnard *et al.*, 2020b; Martin *et al.*, 2020; Debevec *et al.*, 2022b; Steenhorst *et al.*, 2022).

At the level of pulmonary system, few studies reported a blunted HVR in prematurely born adults compared to term born peers (Bates *et al.*, 2014; Debevec *et al.*, 2019), though exercise restored a normal ventilatory response to low O_2 levels (Debevec *et al.*, 2019). The reduced HVR in preterm survivors was attributed to a disruption at the level of the carotid chemoreceptors secondary to neonatal injury from exposure to hyperoxia (Bisgard *et al.*, 2003; Bates *et al.*, 2014). This is consistent with recent observations of reduced HVR in preterm infants already during the second year of life (Freislich *et al.*, 2022). Moreover, as reviewed in *Chapter 2.2.3*, a successful cerebrovascular acclimatization to hypoxia relies on the balance between ventilatory O_2 and CO_2 sensitivity. At this regard, our group has recently demonstrated an increased ventilatory response to severe hypercapnia (6% CO_2 and 21% O_2) in resting preterm adults with reduced HVR compared to term born age-matched participants (Manferdelli *et al.*, 2021). This unbalance between the opposite effects of O_2 and CO_2 on the cerebral vasculature might be critical during acute exposure to hypoxia and leads to an exaggerated vasoconstrictor response or blunted vasodilatory capacity in this population. However, novel findings also suggest greater pulmonary outcomes in preterm adolescents born at the turn of the millennium (compared to their peers born in the last century), and therefore an update of the combined effects of premature birth post-maturation and hypoxia is required. Furthermore, in response to acute normobaric hypoxia (F_IO_2 = 12%), preterm adults with normal baseline cardiac function showed an exaggerated cardiac contractile response, particularly at the level of the RV, which was independent from history of BPD (Barton *et al.*, 2021). In contrast, the hypoxic pulmonary vasoconstrictor response appears intact in preterm children and adults, and similar to term born age-matched individuals (Joshi *et al.*, 2014; Goss *et al.*, 2018a; Laurie *et al.*, 2018). In contrast, the effects of hypoxia on cerebrovascular regulation are unknown in preterm human survivors and limited to a single animal model. In response to acute (30 minutes) hypoxia (F_IO_2 ~10 - 12%) immediately after birth, preterm

lambs showed a marked decrease in middle cerebral artery blood velocity (MCA*v* – index of CBF), together with a drop in the pressor response of similar magnitude (Agrawal et al., 2020). Although very preliminary, these results may suggest a blunted cerebral vasodilatory capacity in response to acute hypoxia in preterm newborns. Furthermore, whether this blunted response in maintained during adulthood remains interrogative.

Most of these specific responses to hypoxia in preterm survivors might be associated with pro-oxidant-to-antioxidant mismatch, in favor of the former. Indeed, both hypoxic and hyperoxic exposures (the second being part of the usual neonatal treatment protocol in preterm newborns) are known to disrupt redox balance by increasing ROS levels within skeletal muscle (**Figure 33**) (Clanton, 2007). Interestingly, despite higher oxidative stress levels in normoxia, adult survivors of premature birth demonstrated lower hypoxia-induced increases in oxidative stress markers compared to their term born peers (Martin *et al.*, 2020), suggesting the development of adaptive mechanisms in the preterm born adult population, limiting ROS overproduction in response to acute exposure to hypoxia, especially during exercise (**Figure 34**).

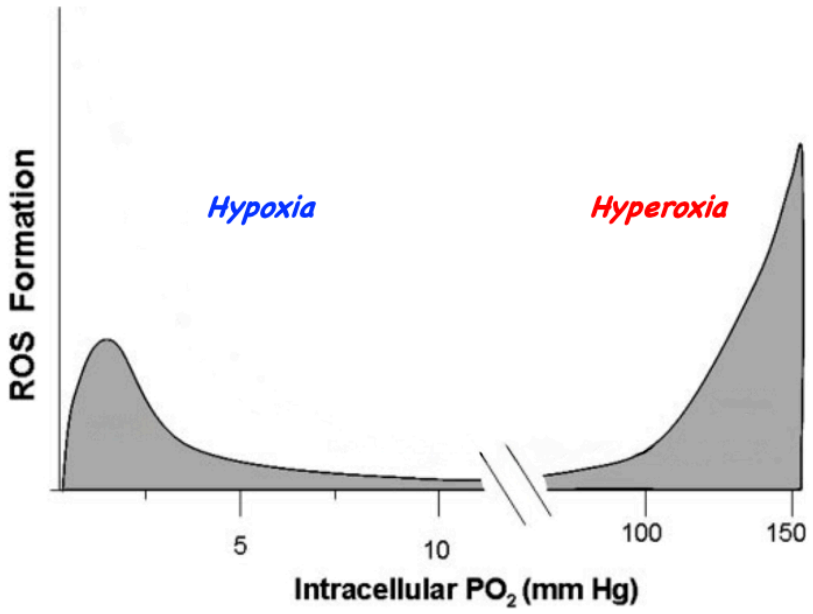

Figure 33. Graphical representation of the bimodal distribution of reactive oxygen species (ROS) production as function of increases (i.e., hyperoxia) or decreases (i.e., hypoxia) in intracellular oxygen partial pressure (PO_2). At rest, normoxic intracellular PO_2 is ~34 mmHg (Richardson *et al.*, 2006). Modified from Clanton (2007).

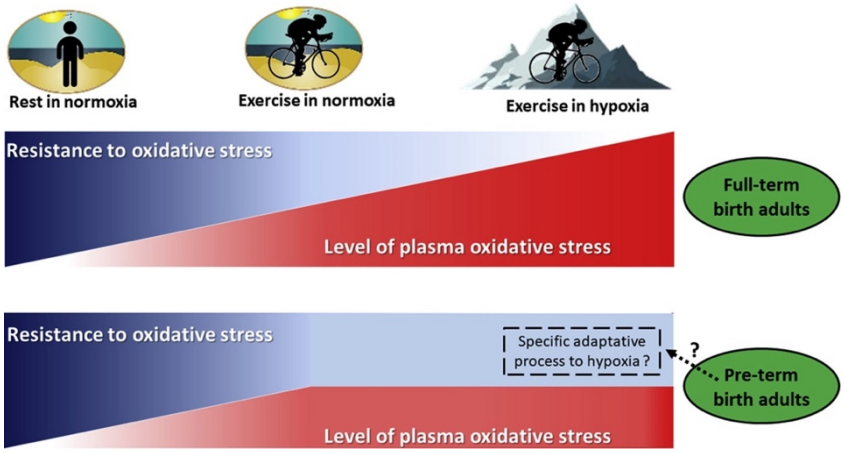

Figure 34. Schematic illustration of oxidative stress levels at rest and during exercise in normoxia and hypoxia in term born and preterm adults. Premature birth seems to confer a favorable oxidative stress profile during hypoxic exposure. For this reason, several studies are currently investigating the hypothesis of a potential "hypoxic preconditioning" effect in preterm adults. Graphical abstract from Martin *et al.* (2020).

However, as oxidative stress is involved in modulating HVR (i.e., higher ROS production leading to greater stimulation of peripheral chemoreceptors) (Pialoux *et al.*, 2009a), lower oxidative stress response to hypoxia may be one underlying mechanism (in addition to carotid chemoreceptors disruption) to explain the blunted HVR previously reported in this population (Bates *et al.*, 2014; Debevec *et al.*, 2019).

Further support of a potential "hypoxic preconditioning" effect of premature birth comes from better maintenance of exercise capacity, despite greater arterial desaturation at volitional exhaustion, in hypoxia in these adults compared to age-matched individuals born at term (Farrell *et al.*, 2015). Also, in the same cohorts, while preterm adults showed wider alveolar-to-arterial O_2 difference at peak exercise in normoxia compared to term born peers, this was not the case anymore during hypoxic exercise (Farrell *et al.*, 2015), suggesting a lower susceptibility to the adverse effects of hypoxia on exercise performance in this group. Similar findings were also reported by our research group where preterm, but not term born, children showed similar $\dot{V}O_{2max}$ in hypoxia compared to normoxia (Narang *et al.*, 2022).

Overall, considering the growing, though still scarse, evidence on the combined effects of preterm birth and hypoxia, one may hypothesize an increased risk for altitude sickness in this

population. However, exercise seems to unmask protective mechanisms against the detrimental effects of hypoxia on exercise performance.

As shown in the following summary figure (**Figure 35**), physiological differences between preterm and term born adults are impacted by the environmental condition (normoxia vs. hypoxia), emphasizing the importance to understand the current open question on a potential "hypoxic preconditioning" effect on the preterm population. Therefore, more research is required to experimentally confirm this speculation and further understand the long-term mechanisms associated to premature birth.

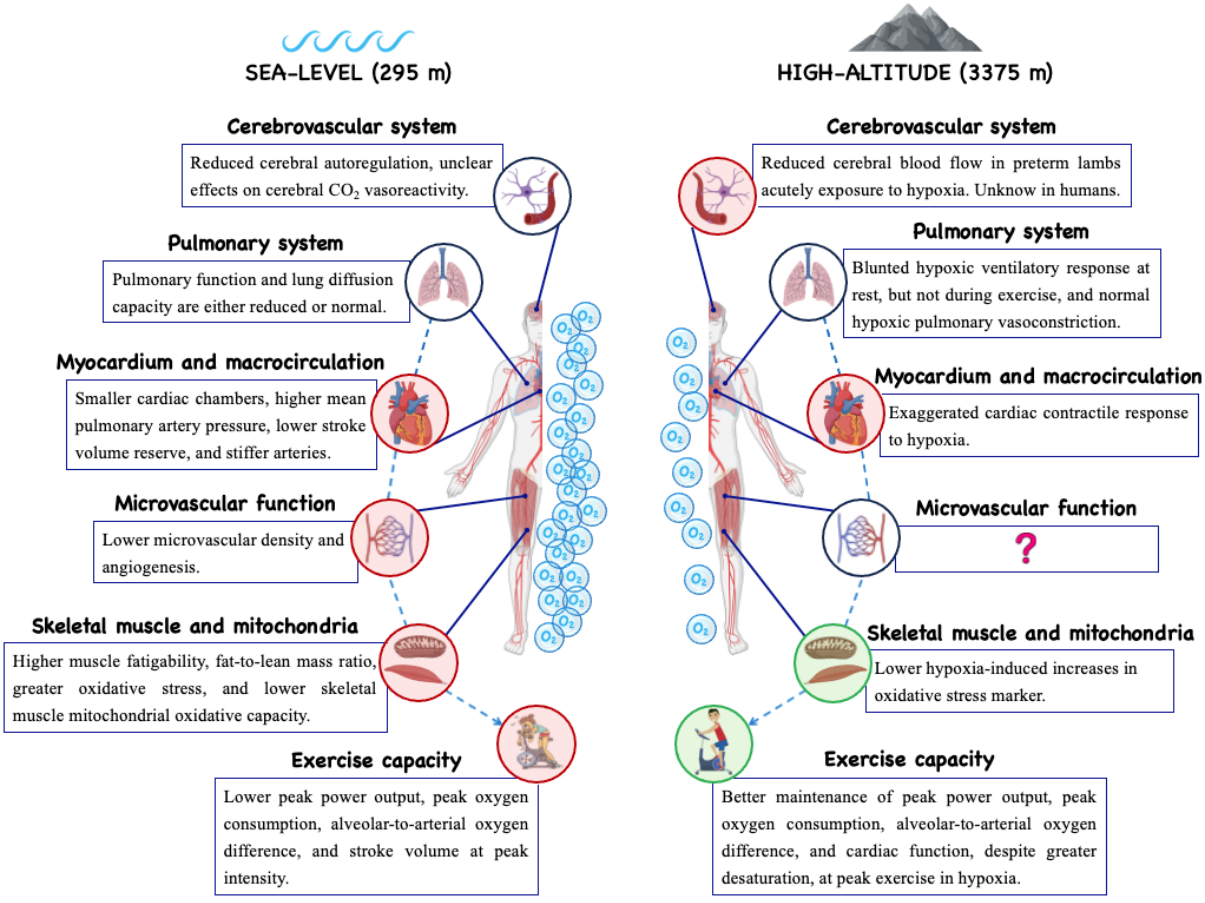

Figure 35. Summary of the existing findings on the physiological responses at rest and during exercise in normoxia and hypoxia in prematurely born individuals compared to their peers born at term. In red, the altered physiological functions described in preterm individuals compared to term born peers; in white those functions on which contrasting findings are currently available; and in green, those responses that are beneficial in preterm vs. term born individuals. The light blue dotted lines and arrows highlights the systems involved in the O_2 cascade and that ultimately determine exercise capacity.

3. Purpose of the thesis and hypotheses

Purpose of the thesis

Based on this review of the literature, we believe that premature birth may induce specific physiological responses to hypoxia, modulate altitude acclimatization, and predispose individuals to an increased risk to high-altitude diseases. Accordingly, the present research project investigated the long-term impact of premature birth on the physiological responses to acute and prolonged exposure to high-altitude, at rest and during exercise. Specifically, this thesis intends to elucidate the mechanisms underlying the cardio- and cerebrovascular, respiratory, muscular, and hematological responses to hypoxia in prematurely born adults compared to term born peers.

The main aims were:

1. To assess the hypoxic ventilatory response in healthy preterm adults and term born peers.
2. To determine the acute effects of exposure to terrestrial altitude (i.e., hypobaric hypoxia) on post-occlusive microvascular reactivity, skeletal muscle oxidative capacity, and oxidative stress in prematurely born, but otherwise healthy, adults and term born age- and fitness-matched peers.
3. To evaluate spontaneous cardiovagal baroreflex sensitivity in prematurely born adults and in control participants born at term, and understand the effects of different combinations of O_2, CO_2, and barometric pressures on spontaneous cardiovagal baroreflex sensitivity in both groups.
4. To investigate the cerebrovascular, ventilatory and cardiovascular responses to CO_2 at both sea-level and high-altitude in prematurely born adults, compared to their term born age-matched peers.
5. To evaluate the full O_2 cascade, and the relative contribution of convective and diffusive components of O_2 transport, during incremental exercise to exhaustion both at sea-level

and after three days of high-altitude sojourn in healthy preterm born adults and age-matched individuals born at term.

6. To determine the effects of prolonged high-altitude exposure (3 days) on oxidative stress, antioxidant biomarkers, and nitric oxide metabolite kinetics in prematurely born adults compared to their term born peers.

Hypotheses

We hypothesized that:

1. The hypoxic ventilatory response would be reduced in prematurely born adults compared to their term born peers.
2. Microvascular reactivity and skeletal muscle oxidative capacity would be impaired in prematurely born adults, and that acute high-altitude exposure would reduce these impairments relative to their term born peers.
3. Spontaneous cardiovagal baroreflex sensitivity in response to hypoxic exposure would be reduced in prematurely born adults compared to term born participants. In addition, due to their higher sensitivity to CO_2, we also hypothesized a stronger effect of breathing additional CO_2 in preterm participant compared to term born controls.
4. Prematurely born adults would display blunted cerebrovascular CO_2 reactivity under normoxia, and furthermore that this phenomenon would be further exacerbated at high-altitude.
5. Exercise capacity and $\dot{V}O_{2peak}$ would be lower in adults born preterm compared to term born peers due to impairments at the convective rather than diffusive O_2 transport level. Moreover, we expected these differences to be exacerbated in hypoxia.

4. Summary of research methodology

Summary of research methodology

4.1 Study design

The overall study design of the project, consisting of three separate data collections, is displayed in **Figure 36**. In this thesis, only the data collected at sea-level and at high-altitude will be displayed and discussed. For the high-altitude phase, participants reached Courmayeur (1300 m) by car, before ascending to the high-altitude location (Torino hut) by cable car. The cable car journey lasted approximately 20 minutes.

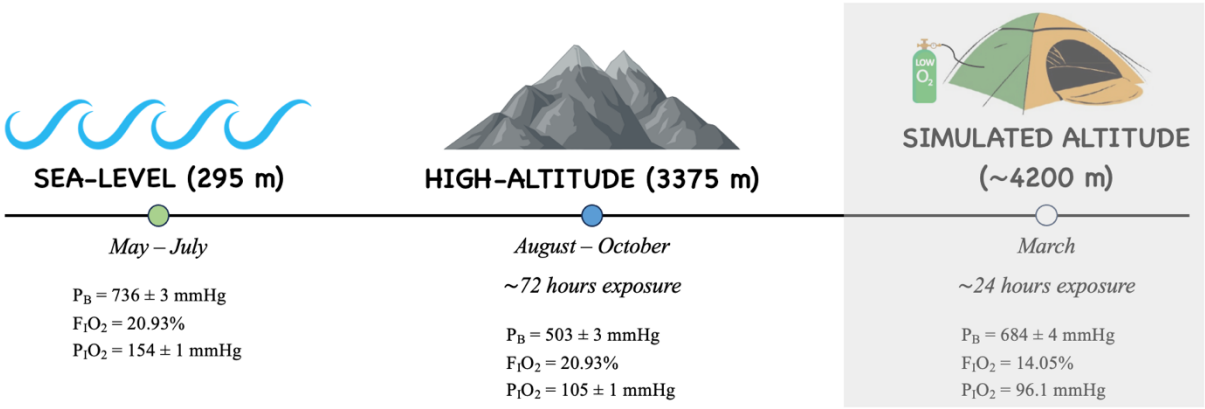

Figure 36. Study design. Participants were tested on three separate visits: near sea-level, during a three-day sojourn at high-altitude (hypobaric hypoxia), and during a 24-hour stay in a hypoxic chamber (normobaric hypoxia). Participants ascended to high-altitude via cable car in ~20 minutes, while in normobaric hypoxia the "ascent" was instantaneous. The grey area on the simulated altitude phase indicates that those results will not be discussed in this thesis. P_B, barometric pressure; F_IO_2, inspired oxygen fraction; P_IO_2, inspired oxygen partial pressure.

4.2 Participants and ethical approval

Forty-one young healthy men volunteered and gave written informed consent to participate in this study. After the initial screening visit, seven participants were excluded due to $\dot{V}O_{2peak}$ > 70 mL·kg^{-1}·min^{-1} and/or contraindications to either intense exercise or exposure to hypoxia. Therefore, a total of thirty-four individuals completed the study protocol. Of these, seventeen

participants were born preterm and seventeen were born at term. The preterm born participants were recruited via the National preterm birth register managed by the University Clinical Centre in Ljubljana, Slovenia using medical record screening and telephone/email-based individual interviews. The inclusion criteria for them were gestational age ≤32 weeks, birth weight ≤1500 g, O_2 therapy at birth, and absence of bronchopulmonary dysplasia diagnosis. The inclusion criteria for the term born participants were gestational age ≥38 weeks and birth weight ≥2500 g. Birth-related inclusion criteria for all participants were checked and confirmed during the initial birth/medical record screening procedure conducted prior to inclusion in the study. Exclusion criteria for all participants comprised of permanent altitude residence (≥1000 m), cardiopulmonary, hematological and/or kidney disorders, chronic medication use, smoking and altitude/hypoxia exposure (≥2000 m) within the last moth prior to the study. Participants were matched for age, height, and body mass. This research project was performed according to the Declaration of Helsinki. The experimental protocol was pre-registered at ClinicalTrials.gov (NCT04739904), and ethical approvals were obtained from both the University of Ljubljana, Faculty of Sport ethics committee (8/2020-316) and the Aosta Hospital Ethical Committee (06/05/2021.0038781.I).

4.3 Pulmonary function and lung diffusion capacity

At sea-level, FVC, FEV_1, FEV_1/FVC ratio, PEF, and maximal voluntary ventilation (MVV) were obtained at rest using a portable spirometer, according to standardized procedures (Graham *et al.*, 2019). Likewise, single breath DL_{CO} and alveolar volume (VA) were assessed in normoxia according to standard procedures (Macintyre *et al.*, 2005). DL_{CO} values were adjusted for individual Hb concentration (Macintyre *et al.*, 2005). Lung function and DL_{CO} parameters were expressed in both absolute terms and as a percentage of predicted values using established equations (Quanjer *et al.*, 2012; Stanojevic *et al.*, 2017).

For more details, *see Chapter 9*, article 1.

4.4 Hypoxic ventilatory response

At sea-level, HVR (taken as an index of hypoxic chemosensitivity) was determined using the pure nitrogen breathing test (Edelman *et al.*, 1973). The experimental setup is shown in **Figure 37**. Briefly, during 10 randomized periods of 1-to-8 consecutive breaths inhaling pure nitrogen, S_pO_2 and $\dot{V}_E$ were continuously measured by pulse oximeter placed on participants' earlobe and a breath-by-breath system (Ergocard Professional, Medisoft, Sorinnes, Belgium), respectively. The lowest measured S_pO_2 value was plotted against the highest $\dot{V}_E$ value obtained within the 30 seconds after each inhalation 100% nitrogen. The slope of this relationship was indicative of the HVR.

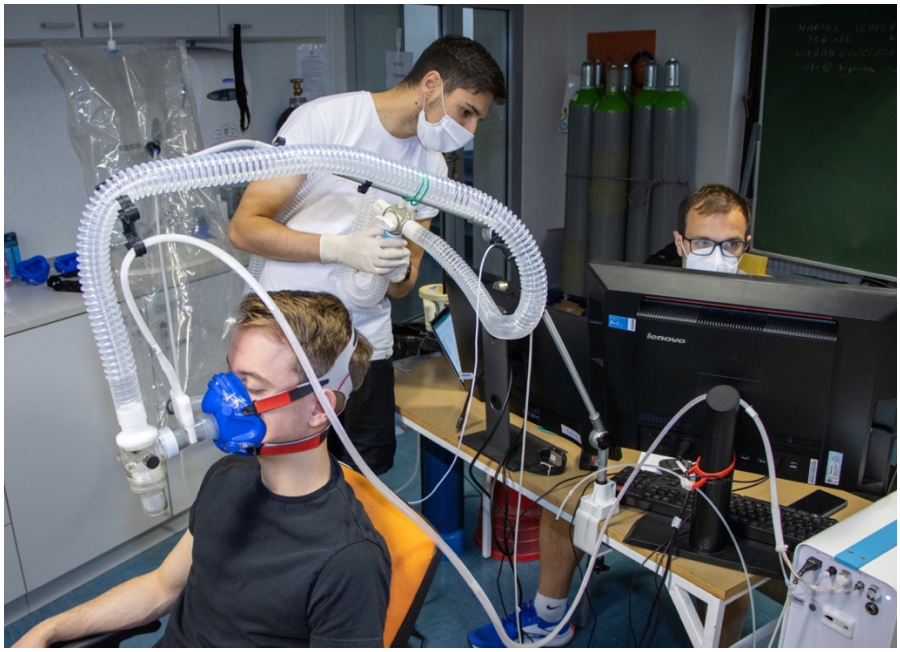

Figure 37. Experimental setup of the pure nitrogen breathing test used to assess hypoxic chemosensitivity in our participants. In the back of the picture, a plastic bag filled with 100% nitrogen was connected via a three-way valve to the mask of the participants. Pulmonary ventilation and peripheral oxygen saturation on the earlobe were continuously measured throughout the test.

For more details, *see Chapter 9*, article 1.

4.5 Acute mountain sickness

The presence and severity of symptoms associated with AMS was assessed approximately 6 h after arrival at high-altitude, as well as on each morning and evening of the following days using the Lake Louise scale (Roach *et al.*, 2018). AMS was diagnosed for scores higher than or equal to 3, in presence of headache.

4.6 Skeletal muscle microvascular reactivity

At sea-level and upon arrival at altitude (within 45 minutes after arrival), skeletal muscle microvascular reactivity was assessed at rest by the vascular occlusion test (VOT) combined with NIRS. Participants rested in seated position on a cycle ergometer before performing a VOT consisting of 5 minutes of tissue ischemia via femoral artery occlusion, followed by 5 minutes of vascular reperfusion (McLay *et al.*, 2016). Arterial occlusion was accomplished using a pneumatic cuff placed on the proximal part of the thigh and connected to an automatic rapid inflation system (HokansonE20 AG101, Bellevue, WA, USA). Occlusion pressure was set between 290 and 300 mmHg, maintained for the entire 5-minute of ischemia, and it was identical at sea-level and at altitude. Oxygenation changes, assessed as changes in tissue saturation index (TSI), in the *vastus lateralis* muscle were evaluated by a continuous wave NIRS device (Portamon, Artinis Medical Systems, Elst, The Netherlands). Several parameters were used for analyses (Rasica *et al.*, 2021a), including (**Figure 38**): *a*) baseline TSI as the 30 s average before cuff occlusion; *b*) the linear regression of TSI signal during the first minute of occlusion (desaturation rate) as an index of resting skeletal muscle oxidative metabolism; *c*) the upslope of the TSI signal during the first 10 s following cuff release (reperfusion rate); *d*) the time required for the TSI signal to return to baseline values after cuff release ($t_{baseline}$); and *e*) the area under the reperfusion curve above baseline TSI until 2 minutes post cuff release (AUC_{2MIN}).

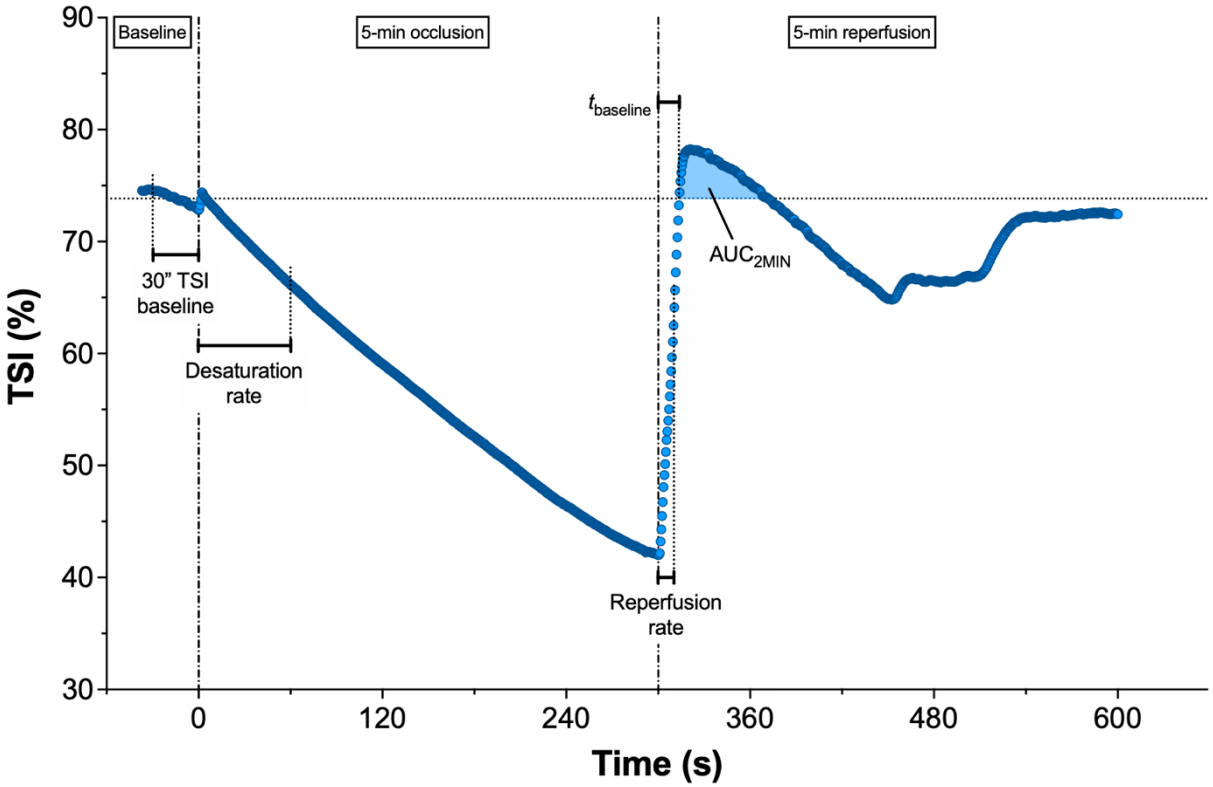

Figure 38. Graphical representation of changes in tissue saturation index (TSI) during a vascular occlusion test (VOT) in a typical participant. Vertical dashed lines indicate pneumatic cuff inflation (occlusion phase) and deflation (reperfusion phase), while the horizontal dotted line represents baseline TSI. Some of the investigated NIRS-VOT parameters used for analyses are also shown. AUC_{2MIN}, area under the curve above baseline TSI during the first 2 minutes of reperfusion; $t_{baseline}$, time required for the TSI signal to return to baseline values after cuff release.

For more details, *see Chapter 10*, article 2.

4.7 Skeletal muscle oxydative capacity and oxygen diffusion capacity

At sea-level and during acute exposure to hypoxia (within 90 minutes after arrival), oxidative capacity in the *vastus lateralis* muscle was evaluated *in vivo* by measuring post-exercise muscle O_2 consumption ($m\dot{V}O_2$) recovery kinetics by NIRS and the repeated occlusions method (Adami & Rossiter, 2018). The protocol consisted of a 10-minute moderate intensity (~50% $\dot{V}O_{2peak}$) constant work rate exercise bout aiming to increase $m\dot{V}O_2$, which was immediately followed by a series of 5-s intermittent arterial occlusions (5 separated by a 5-s cuff release, 5

separated by a 10-s release, and 5 separated by a 20-s release). Occlusion pressure was set between 290-300 mmHg and it was kept the same between the two conditions. For each intermittent arterial occlusion, the rate of decline in TSI (expressed in $\%\cdot s^{-1}$) was fitted by a linear function to estimate relative $m\dot{V}O_2$ (Adami *et al.*, 2020). $m\dot{V}O_2$ values were then fitted by a monoexponential function and τ, which represents is the rate constant ($k = [1/\tau]$, expressed in min^{-1}) of the function, was determined. The exponential $m\dot{V}O_2$ recovery rate constant (k, min^{-1}) was taken as an estimate of skeletal muscle oxidative capacity. The TSI trace during the repeated occlusions protocol and the $m\dot{V}O_2$ recovery kinetics in a typical participant are shown in **Figure 39**. Moreover, DmO_2 was estimated as the absolute difference between k obtained under normoxic and hypoxic conditions (Manferdelli *et al.*, 2022; Pilotto *et al.*, 2022).

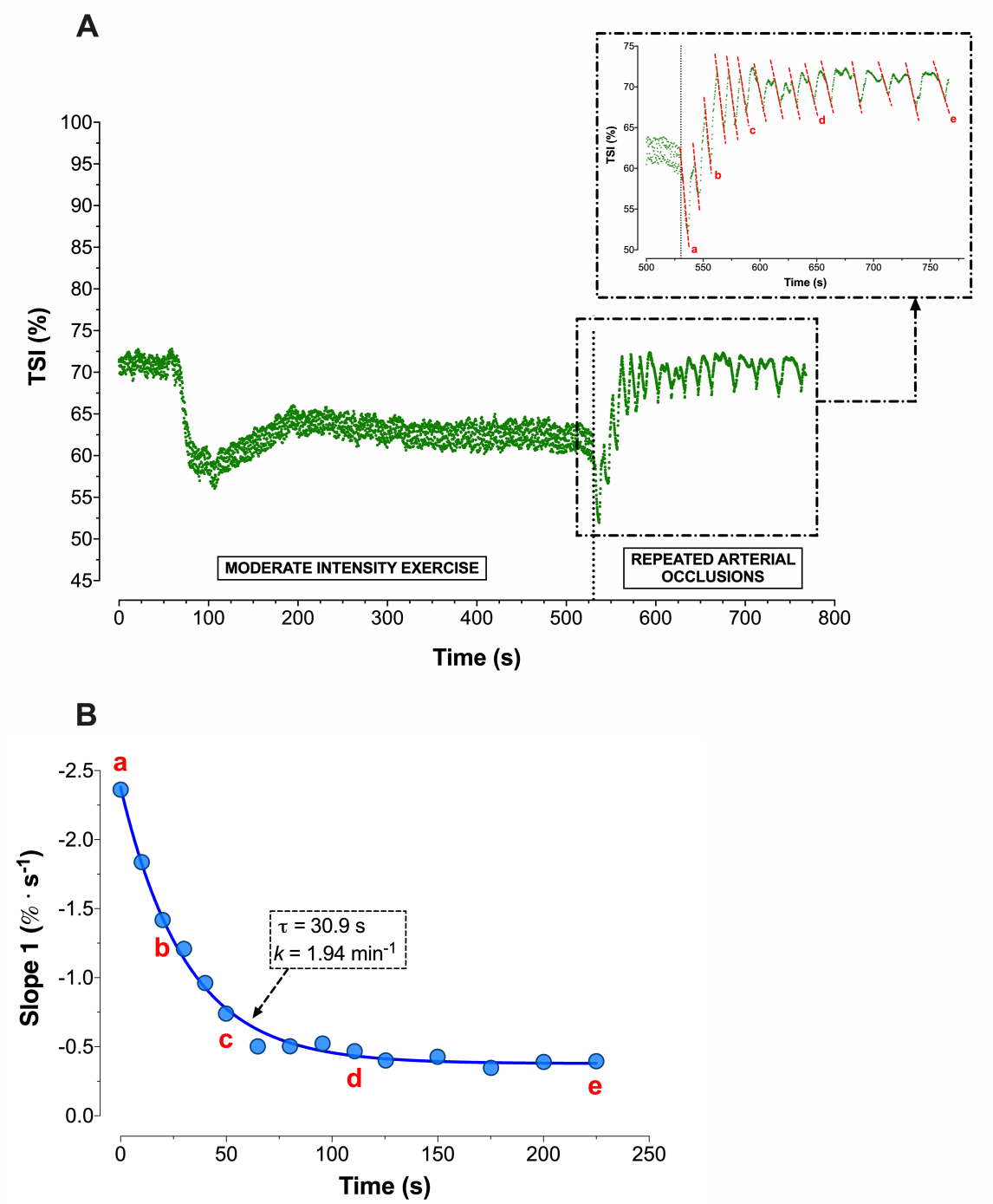

Figure 39. Representative TSI trace during the muscle oxidative capacity assessment. Panel A illustrates the TSI dynamics during moderate intensity exercise followed by a series of intermittent arterial occlusions during recovery. The panel on the upper right corner shows the TSI changes during intermittent arterial occlusions and the red dotted lines represent the linear regression during each occlusion. Panel B depicts the slopes of each occlusion and the calculated muscle $\dot{V}O_2$ ($m\dot{V}O_2$) recovery profiles (dashed line). *k* represents the rate constant, which is linearly related to muscle oxidative capacity ($k = (1/\tau)\cdot 60$, min^{-1}. The letters (a–e) illustrate how the corresponding $m\dot{V}O_2$ value is derived from respective TSI negative slopes during intermittent occlusions.

For more details, *see Chapter 10*, article 2.

4.8 Baroreflex sensitivity

BRS was measured at rest with the participant comfortably seated on a chair. Both at sea-level and at high-altitude, all experiments were conducted in the morning at the same time of the day, with high-altitude testing performed in the first morning after arrival at altitude.

Participants underwent six 6-minute stages of continuous BP (by finger photopletismography - NIBP100D, Biopac Systems Inc., Goleta, CA, USA), heart rate (by finger photopletismography), and $P_{ET}CO_2$ (by a breath-by-breath system) recording in the following order: (1) normobaric normoxia (NNx), (2) normobaric normoxia hypercapnia (NNx+CO_2), (3) hypobaric hypoxia (HHx), (4) hypobaric normoxia (HNx), (5) hypobaric normoxia hypercapnia (HNx+CO_2), (6) hypobaric hypoxia with $P_{ET}CO_2$ clamped at NNx value (HHx+clamp). Normobaric conditions were performed in Ljubljana, while hypobaric measurements were carried out at high-altitude. NNx+CO_2 was induced by switching the inspired gas from ambient air to 3% CO_2 (in 20.93% O_2, balance N_2). In HNx and HNx+CO_2, participants breathed supplemental O_2 (FiO_2 = 32%, with 0.03% CO_2, balance N_2 and with 3% CO_2, balance N_2, respectively). Phases 1 and 2 were performed at sea-level, while stages 3-6 at high-altitude. BRS was determined using the sequence method (La Rovere *et al.*, 2008). A schematic overview of the experimental protocol is illustrated in **Figure 40**.

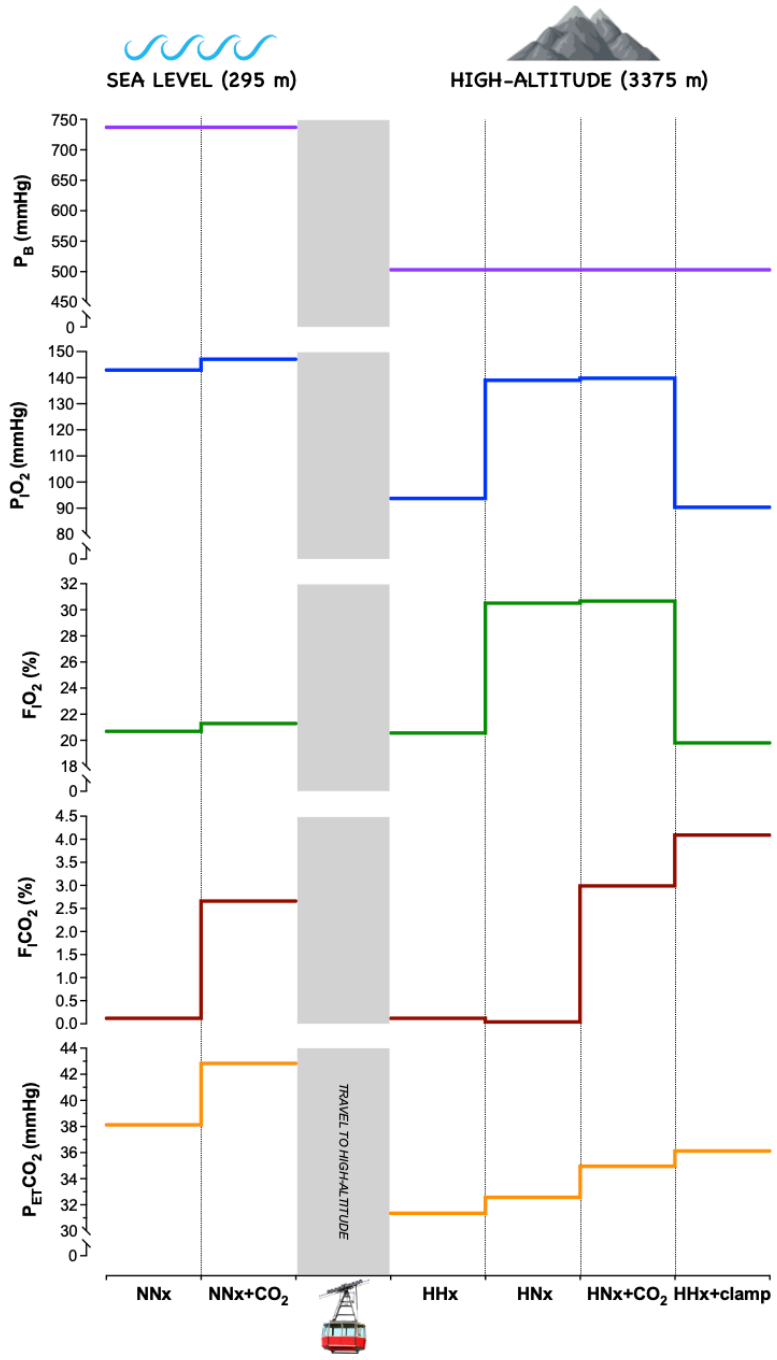

Figure 40. Schematic representation of the experimental protocol used to investigate the effects of different barometric, oxygen, and carbon dioxide pressures on spontaneous cardiovagal baroreflex sensitivity in term born and preterm adults. The protocol consisted of six 6-minute stages. Of these, the first two conditions (NNx and NNx+CO_2) were conducted near sea-level, while the remaining four stage ~ 20 hours after arrival at high-altitude. *NNx*, normobaric normoxia; *NNx+CO_2*, normobaric normoxia hypercapnia; *HHx*, hypobaric hypoxia; *HNx*, hypobaric normoxia; *HNx+CO_2*, hypobaric normoxia hypercapnia; *HHx+clamp*, hypobaric hypoxia with $P_{ET}CO_2$ clamped at NNx value; P_B, barometric pressure; P_IO_2, inspired oxygen partial pressure; F_IO_2, inspired fraction of oxygen; F_ICO_2, inspired fraction of carbon dioxide; $P_{ET}CO_2$, end-tidal partial pressure of carbon dioxide.

For more details, *see Chapter 11*, article 3.

4.9 Cerebrovascular reactivity to carbon dioxide

Both at sea-level and on the second morning at high-altitude, participants were exposed to two consecutive 4-minute hyperoxic hypercapnic conditions (3%CO_2-97%O_2; 6%CO_2-94%O_2), followed by two 4-minute periods of voluntary hyperventilation-induced hypocapnia. MCA*v* (by transcranial doppler - ST3, Spencer technology, Seattle, WA, USA), beat-by-beat MAP (by finger photopletismography), $P_{ET}CO_2$ and $\dot{V}_E$ (by a breath-by-breath system) were continuously measured during the test. **Figure 41** illustrates the experimental setup. Cerebrovascular CO_2 reactivity was determined by fitting a sigmoid curve on the MCA*v* - $P_{ET}CO_2$ relationship (Fan *et al.*, 2015).

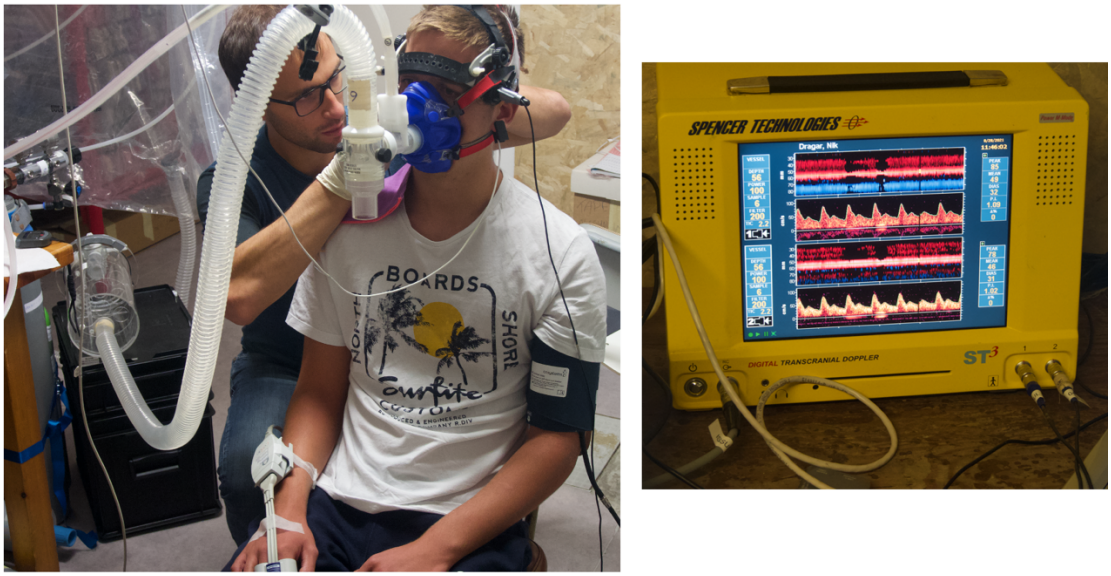

Figure 41. Experimental setup during the cerebrovascular carbon dioxide reactivity protocol (left panel) and middle cerebral artery blood velocity trace (right panel) during the test. A bag was filled with each gas mixture and transition from one gas mixture to another one was accomplished in a progressive manner using a mixing chamber. Cerebral blood flow velocity in the middle cerebral artery velocity was measured via transducers place on the right and left temples of the participants. A capillary blood sample was taken at the end of each stage from the participants' earlobe to measure arterial blood gases.

For more details, *see Chapter 12*, article 4.

4.10 Exercise capacity: convective and diffusive mechanisms of oxygen transport

Gas exchange, cardiac hemodynamics, and both muscle and cerebral oxygenation were assessed using a breath-by-breath system, transthoracic impedance (Physioflow Enduro, Manatec Biomedical, Paris, France), and NIRS (Portamon and Portalite, Artinis Medical Systems, Elst, The Netherlands), respectively, during an incremental cycling exercise test to exhaustion performed at sea-level and after three days of high-altitude exposure. After 5 minutes of seated rest on an electrically-braked cycle ergometer (Premium 8i ergo bike, Daum Electronic gmbh, Fürth, Germany), participants pedaled at 60W for 2 minutes, afterward power was increased by 40W every 2 minutes until volitional exhaustion. Throughout the tests, participants were instructed to maintain a pedaling frequency between 75 and 85 rpm. Maximal exhaustion was considered attained when at least three of the following parameters were observed: capillary lactate concentration ($[La^-]_b$) >8 $mmol \cdot L^{-1}$ within 1 minute after exercise cessation, a respiratory exchange ratio >1.10, a maximal heart rate (HR) >85% of the participant's predicted maximal HR, or a rating of perceived exertion higher than 18 using the Borg's 6-20 scale (Borg, 1982).

As recently proposed and discussed (Manferdelli *et al.*, 2023a, b; Porcelli *et al.*, 2023a, b), $\dot{Q}O_2$ and DO_2 mechanisms of O_2 transport were analyzed using a modified version of graphical model proposed by Wagner (Wagner, 1993). Briefly, given that a consistent number of investigations on both humans and animal models demonstrated a high correlation between NIRS signals and mean venous O_2 saturation under different blood flow rates, F_IO_2, and exercise intensities (Vogiatzis *et al.*, 2015; Sun *et al.*, 2016), we assumed NIRS-derived muscle TSI to be a relevant substitute to mean $PcapO_2$ for illustrative purposes (Goulding *et al.*, 2021), and therefore taken as an index of P_vO_2 (Manferdelli *et al.*, 2023a, b).

For more details, *see Chapter 9*, article 1.

4.11 Plasma biomarkers of oxidative stress and nitric oxide

At sea-level, immediately after arrival at altitude, and on each morning at high-altitude upon awake (day 1, day 2, and day 3), 6 mL of venous blood were obtained from the antecubital vein and immediately frozen at -80°C. Plasma levels of oxidative stress markers [AOPP, xanthine oxidase (XO) activity, malondialdehyde (MDA), and myeloperoxidase (MPO) activity], antioxidant enzymes [Catalase activity, Glutathione peroxidase (GPx) activity, SOD activity], antioxidant non-enzymatic markers [ferric reducing antioxidant power (FRAP)], and NO metabolites [nitrite (NO_2^-) and total nitrite and nitrate (NOx)] were analyzed. Biochemical analyses of plasma oxidative stress markers and NO metabolites were performed as previously described elsewhere (Martin *et al.*, 2020; Raberin *et al.*, 2021).

For more details, *see Chapter 13*, article 5.

5. Summary of experimental results

Summary of experimental results

In this section, the main findings discussed in 5 papers published, accepted for publication, or currently under revision in scientific journals (*see Chapters 9 - 13*) are presented.

Table 1 shows participants' characteristics and pulmonary function assessed at sea-level.

Table 1. Participants' baseline characteristics and pulmonary function assessed at sea-level. Data are reported as Mean ± SD.

	Term born (*n* = 17)	**Preterm (*n* = 17)**	***P*-value**
Participants' characteristics			
Gestational age (weeks)	40 ± 0	29 ± 1	***P* < 0.001**
Birth weight (g)	3621 ± 101	1132 ± 64	***P* < 0.001**
Age (years)	21 ± 1	21 ± 1	*P* = 0.066
Height (cm)	182 ± 2	178 ± 2	*P* = 0.210
Body mass (kg)	75.6 ± 1.7	72.4 ± 3.5	*P* = 0.415
BMI ($kg \cdot m^{-2}$)	22.8 ± 0.4	22.5 ± 0.7	*P* = 0.713
Body surface area (m^2)	1.95 ± 0.03	1.89 ± 0.06	*P* = 0.322
Pulmonary function			
FVC (L)	5.67 ± 0.17	5.37 ± 0.31	*P* = 0.285
FVC (%predicted)[1]	98 ± 2	98 ± 3	*P* = 0.809
FEV_1 (L)	4.63 ± 0.18	4.23 ± 0.13	*P* = 0.082
FEV_1 (%predicted)[1]	93 ± 2	92 ± 2	*P* = 0.412
FEV_1/FVC	0.82 ± 0.02	0.79 ± 0.01	*P* = 0.261
FEV_1/FVC (%predicted)[1]	92 ± 3	88 ± 5	*P* = 0.167
PEF ($L \cdot s^{-1}$)	8.69 ± 0.37	8.83 ± 0.34	*P* = 0.792
MVV ($L \cdot min^{-1}$)	195.0 ± 7.9	180.4 ± 6.9	*P* = 0.172
MVV (%predicted)[1]	106 ± 3	107 ± 3	*P* = 0.820
DL_{CO} ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	35.1 ± 0.8[2]	31.8 ± 1.9[3]	*P* = 0.121
DL_{CO} (%predicted)[1]	102 ± 3[2]	99 ± 5[3]	*P* = 0.554
VA (L)	7.1 ± 0.2[2]	6.5 ± 0.3[3]	*P* = 0.072
VA (%predicted)[1]	106 ± 2[2]	105 ± 3[3]	*P* = 0.942
DL_{CO}/VA	5.0 ± 0.2[2]	4.9 ± 0.2[3]	*P* = 0.671
DL_{CO}/VA (%predicted)[1]	97 ± 3[2]	93 ± 3[3]	*P* = 0.453

BMI, body mass index; *FVC*, forced vital capacity; FEV_1, forced expiratory volume in 1 second; *PEF*, peak expiratory flow; *MVV*, maximal voluntary ventilation; DL_{CO}, lung diffusive capacity for carbon monoxide; *VA*, alveolar volume.

[1]predicted values using the ATS/ERS equations (Quanjer *et al.*, 2012; Stanojevic *et al.*, 2017).

[2]n-1.

[3]n-2.

In average, the preterm group investigated in the present research project included extremely-to-very preterm born adults. Participants were well-matched for age, BMI, and body surface area. Lung function and D_LCO were also similar between preterm and term born adults.

For more details, *see Chapter 9*, article 1.

AMS prevalence and severity were similar between preterm and term born participants 6 h after arrival at altitude (4 term born and 4 preterm). On the first morning, AMS symptoms were diagnosed in 2 term born and 4 preterm adults. On the following time point, AMS was not diagnosed anymore.

5.1 Chemosensitivity to hypoxia is similar between preterm and term born adults

Figure 42 shows the relationship between $\dot{V}_E$ and S_pO_2 during the pure nitrogen breathing test in a typical participant (panel A), and the group means (±SD) and individual values for the HVR. The maximum absolute change in S_pO_2 and $\dot{V}_E$ during the test from resting values were also similar between preterm and term born participants (28 ± 13% vs. 31 ± 12% and 11.1 ± 6.1 vs. 10.5 ± 4.8 $L \cdot min^{-1}$, $P = 0.554$ and $P = 0.771$, respectively).

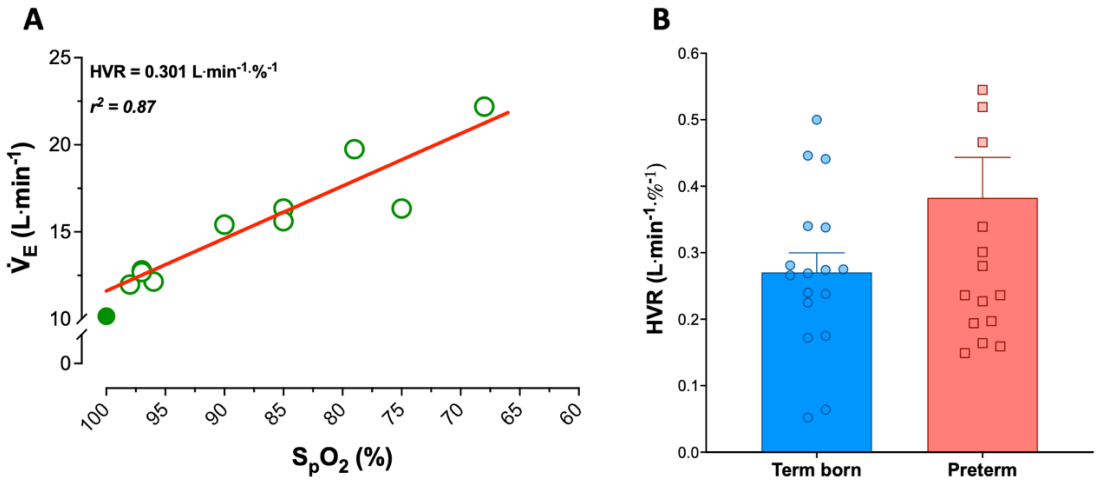

Figure 42. Typical representation of the change in pulmonary ventilation ($\dot{V}_E$) in response to changes in peripheral oxygen saturation (S_pO_2) in one of the participants investigated (Panel A). The filled circle represents baseline values, while empty circles the ventilatory and S_pO_2 values recorded during each transient period of pure nitrogen breathing. The red line represents the slope of this relationship and taken as index of hypoxic chemosensitivity. In Panel B, group means (± SD) and individual values for the hypoxic ventilatory response (HVR).

For more details, *see Chapter 9*, article 1.

5.2 Blunted microvascular responsiveness, larger increases in oxidative stress and skeletal muscle oxidative capacity in response to acute exposure to hypoxia in preterm compared to term born adult participants

Upon acute altitude exposure, compared to sea-level, microvascular reperfusion rate was lower in preterm (7 ± 31%) compared to term born (30 ± 30%, $P = 0.046$) participants (**Figure 43**), while k was higher in the former group compared to controls (6 ± 32% vs. -15 ± 21%, $P = 0.039$). The altitude-induced increases in plasma AOPP and catalase activity were higher (35 ± 61% vs. -13 ± 48% and 67 ± 64% vs. 15 ± 61%, $P = 0.034$ and $P = 0.010$, respectively) and in XO were lower (29 ± 82% vs. 159 ± 162%, $P = 0.030$) in preterm compared to term born adults, suggesting greater hypoxia-induced oxidative stress in the former group.

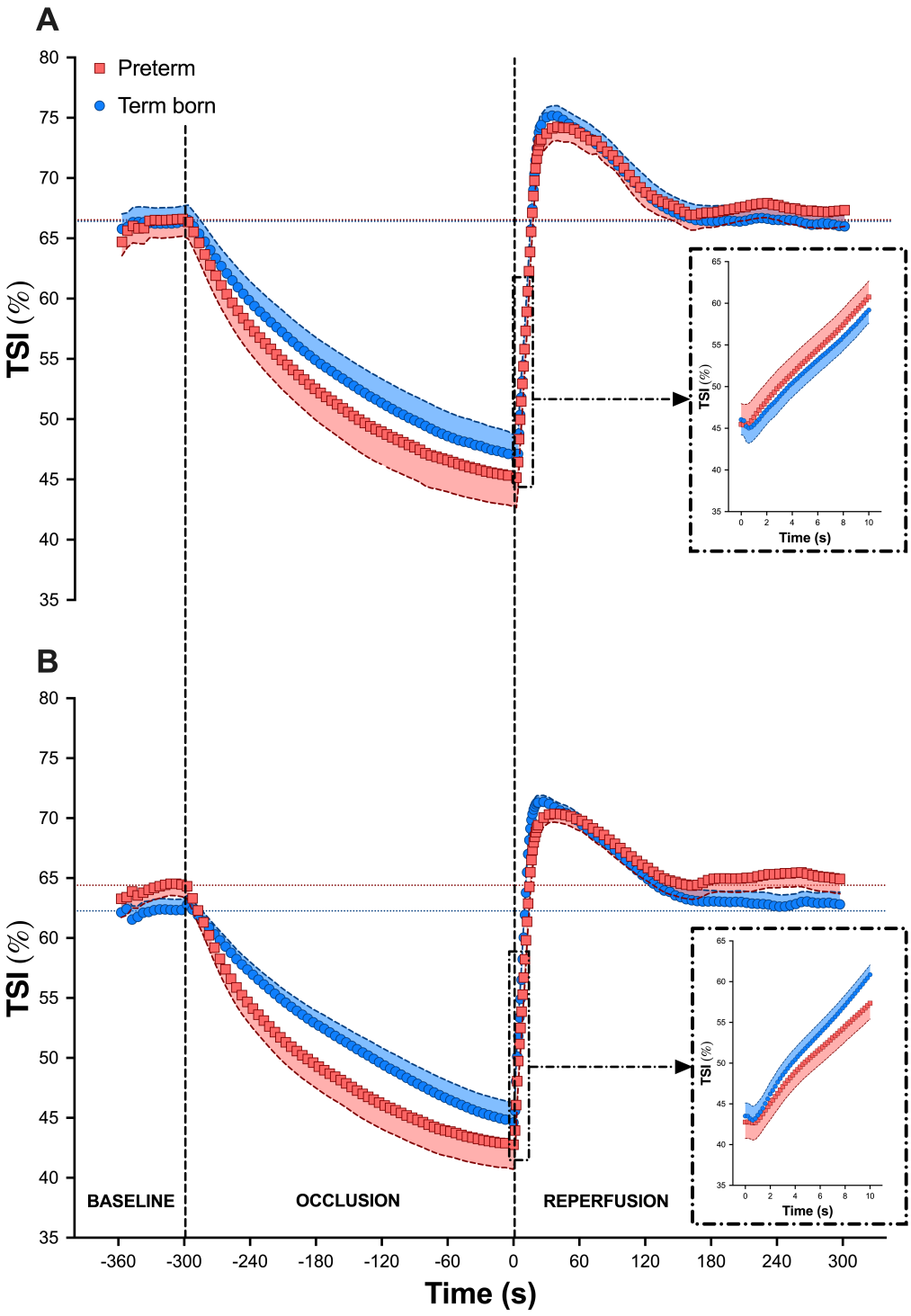

Figure 43. Group means (± SEM) of oxygen saturation signal (TSI) during the vascular occlusion test performed at sea-level (panel A) and upon arrival at high-altitude (3375 m; panel B) in prematurely born (red squares) and term born (blue circles) adults. Vertical dashed lines indicate instances of cuff inflation and deflation, while horizontal dashed lines indicate baseline values for each group. For each condition, the lower panel on the right side depicts the mean data during the first 10 seconds after cuff release (reperfusion rate).

For more details, *see Chapter 10*, article 2.

5.3 Hypoxia-induced changes in spontaneous cardiovagal baroreflex sensitivity differ between preterm and term born adults

In all participants, P_IO_2 was matched between NNx vs. HNx vs. HNx+CO_2 (142.9 ± 0.5 vs. 139.0 ± 3.7 vs. 139.8 ± 2.5 mmHg, respectively). Likewise, $P_{ET}CO_2$ was matched between NNx and HHx+clamp (38 ± 3 vs. 36 ± 3 mmHg, respectively). **Figure 44** depicts BRS in both term born and preterm adults during stages 1-6 of the experimental protocol. Despite similar cardiorespiratory fitness (**Table 1**), BRS was lower in term born compared to preterm (13.0 ± 7.5 vs. 21.2 ± 8.8 ms·mmHg^{-1}, main group effect: $P < 0.01$) participants across all conditions. BRS was lower in HHx compared to NNx in term born (10.5 ± 4.9 vs. 16.0 ± 6.0 ms·mmHg^{-1}, $P = 0.05$), but not in preterm (27.3 ± 15.7 vs. 17.6 ± 8.3 ms·mmHg^{-1}, $P = 0.43$) participants, leading to a lower BRS in HHx in term born compared to preterm ($P < 0.01$) adults. Interestingly, no between-group difference in systolic BP, heart rate, and respiratory parameters were observed in any of the conditions.

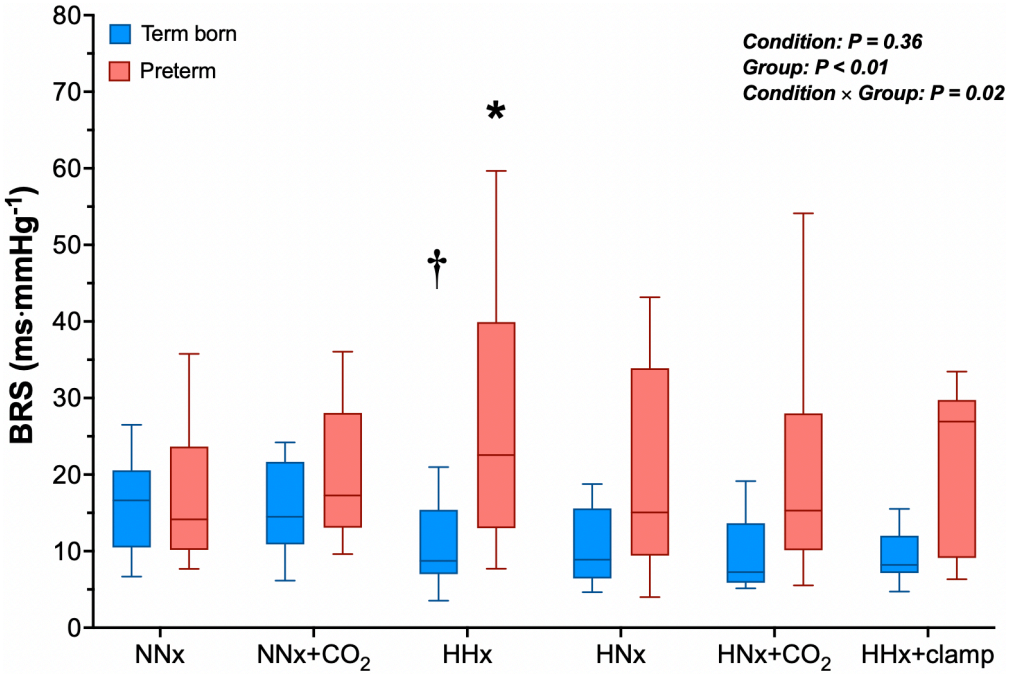

Figure 44. Tukey boxplots of baroreflex sensitivity (BRS) in term born (in blue) and prematurely born (in red) adults during the 6 stages of the experimental protocol. Horizontal line inside boxes represents median; upper and

lower lines of boxes the 75th and 25th percentiles, respectively; upper and lower whiskers the highest and lowest data points within the 1.5 inter quartile range. NNx, normobaric normoxia; NNx+CO_2, normobaric normoxia with 3% CO_2; HHx, hypobaric hypoxia (3375 m); HNx, hypobaric normoxia; HNx+CO_2, hypobaric normoxia with 3% CO_2; HHx+clamp, hypobaric hypoxia with $P_{ET}CO_2$ clamped at NNx value. *Different from term born; †different from NNx.

For more details, *see Chapter 11*, article 3.

5.4 Cerebrovascular CO_2 reactivity is impaired at high-altitude in preterm born adults

A typical MCA*v* response to changes in $P_{ET}CO_2$ both at sea-level (Panel A and C) and at high-altitude (Panel B and D) in a term born and preterm participant is shown in **Figure 45**. While at sea-level MCA*v* was similar between term born and preterm participants (48.5 ± 9.9 vs. 53.3 ± 9.8 $cm \cdot s^{-1}$, $P = 0.295$), altitude exposure increased MCA*v* in term born (+24 ± 39%, $P = 0.035$) but not in preterm (-4 ± 27%, $P = 0.295$) participants leading to a significantly higher MCA*v* in term born compared to preterm adults ($P = 0.046$).

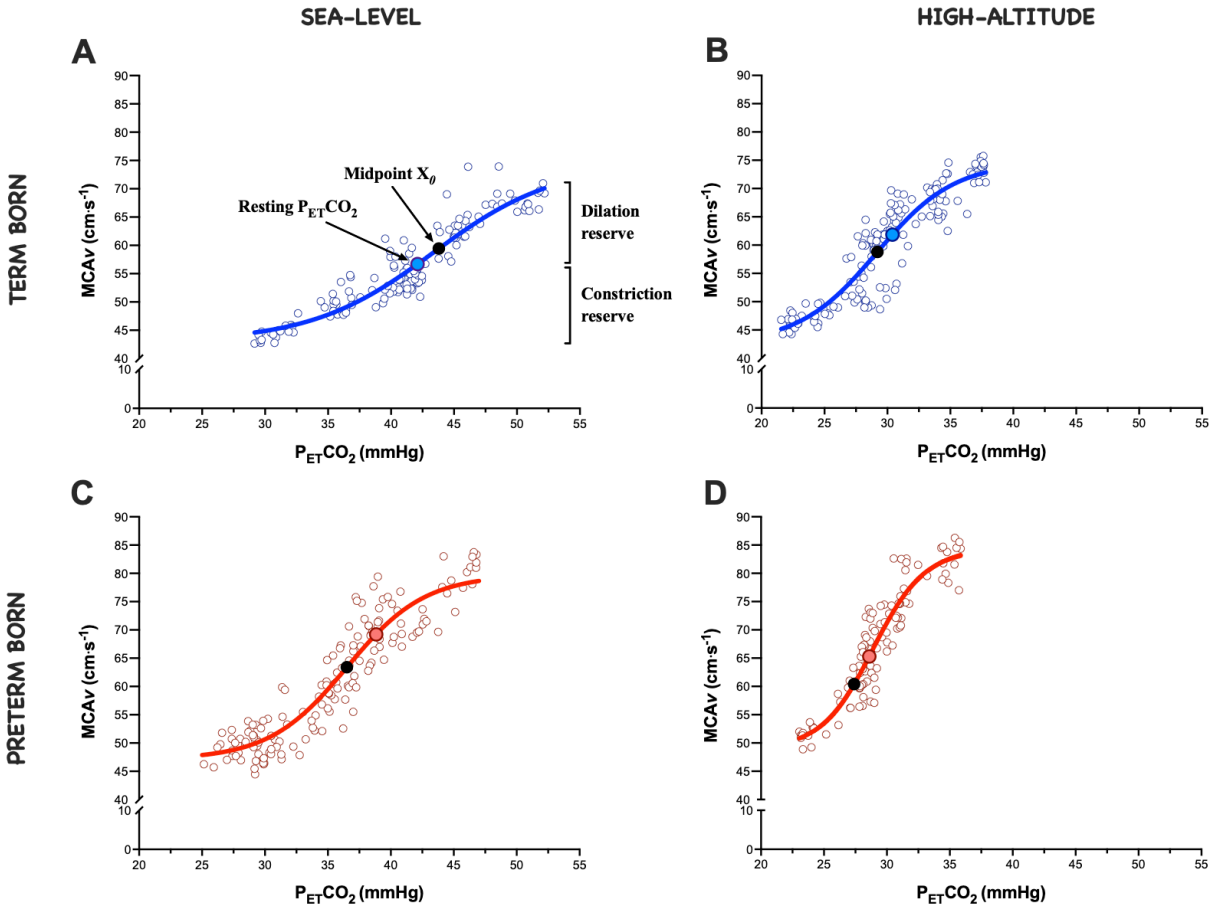

Figure 45. Changes in middle cerebral artery velocity (MCA*v*) in response to changes in end-tidal carbon dioxide partial pressure ($P_{ET}CO_2$) in typical term born (upper panels, blue dots and lines) and preterm born (lower panels, red dots and lines) participants at sea-level (panels A and C, respectively) and at high-altitude (3375 m; panels B and D, respectively). The MCA*v*-$P_{ET}CO_2$ relationship was fit with a sigmoid curve (solid lines). The midpoint X_0 (black dot) of the sigmoid curve represents the optimal operating point of the vessels' capacity to dilate and constrict. Resting $P_{ET}CO_2$ represents baseline values of MCA*v* and $P_{ET}CO_2$ while breathing ambient air.

Relative hypercapnic and hypocapnic cerebrovascular reactivity is shown in **Figure 46**. Hypercapnic cerebrovascular reactivity was similar between term born and preterm participants at sea-level (4.7 ± 1.8 vs. 3.5 ± 2.1 %·mmHg^{-1}, $P = 0.461$), while, at high-altitude, it was significantly lower in term born compared to preterm participants (4.4 ± 2.0 vs. 7.5 ± 4.7 %·mmHg^{-1}, $P = 0.012$). In contrast, hypocapnic cerebrovascular reactivity at high-altitude was significantly higher in term born compared to preterm participants (3.9 ± 2.2 vs. 2.1 ± 2.6 %·mmHg^{-1}, $P = 0.012$).

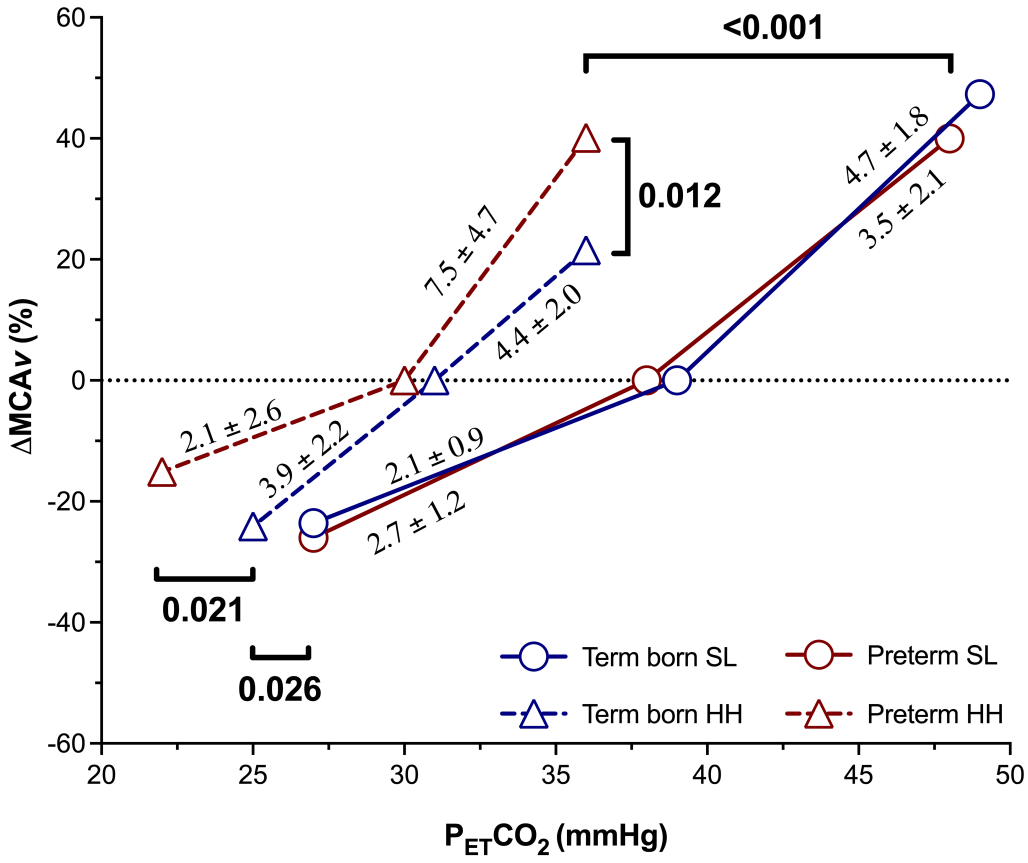

Figure 46. Hypercapnic and hypocapnic cerebrovascular reactivity at sea-level (SL; circles) and at high-altitude (HH; 3375 m; triangles) in term born (blue) and preterm born (red) participants. Hypercapnic and hypocapnic cerebrovascular reactivity was determined as the percent change in middle cerebral artery velocity from baseline during a 4-minute steady-state period breathing an hyperoxic hypercapnic gas mixture (6%CO_2 in 94%O_2) and a 4-minute voluntary hyperventilation (to induce hypocapnia) phase, respectively, per unit change in end-tidal carbon dioxide partial pressure (%·mmHg^{-1}).

The amplitude of the MCA*v* response was similar between groups (term born: 109.9 ± 19.2 cm·s^{-1}; preterm: 109.4 ± 20.0 cm·s^{-1}, $P = 0.948$) and altitudes (sea-level: 109.2 ± 21.8 cm·s^{-1}; high-altitude: 110.1 ± 16.9 cm·s^{-1}, $P = 0.832$). X_0, which is indicative of the optimal operating point of the vessels' capacity to dilate and constrict, was higher at sea-level compared to high-altitude (40 ± 3 vs. 30 ± 2 mmHg, respectively, $P < 0.001$) and in term born adults compared to preterm participants (36 ± 6 vs. 35 ± 5 mmHg, $P = 0.025$). No between-group difference in cardiovascular and ventilatory parameters were observed both at sea-level and at high-altitude.

For more details, *see Chapter 12*, article 4.

5.5 Exercise capacity in preterm adults is impaired by reduced convective rather than diffusive mechanisms involved in the O_2 transport

In normoxia, preterm demonstrated lower peak power output (276 ± 10 vs. 312 ± 12 W, P = 0.042) compared to term born adults, but not in hypoxia (248 ± 10 vs. 279 ± 10 W, P = 0.070), despite similar relative $\dot{V}O_{2peak}$ in both normoxia (48.5 ± 2.6 vs. 51.9 ± 1.9 mL·kg^{-1}·min^{-1}, P = 0.358) and hypoxia (36.4 ± 1.6 vs. 37.9 ± 1.1 mL·kg^{-1}·min^{-1}, P = 0.873). At peak, stroke volume (61 ± 3 vs. 71 ± 3 mL·m^{-2}, P = 0.020) and $\dot{Q}$ (11.4 ± 0.5 vs. 13.3 ± 0.5 L·min^{-1}·m^{-2}, P = 0.008), both indexed to body surface area, were lower in preterm compared to term born participants in normoxia, but not in hypoxia (61 ± 3 vs. 61 ± 2 mL·m^{-2}, P = 0.983 and 11.2 ± 0.5 vs. 11.6 ± 0.4 L·min^{-1}·m^{-2}, P = 0.835). Peak Δ[deoxy(Hb+Mb)], which reflects skeletal muscle O_2 extraction, increased from normoxia to hypoxia only in term born (11.04 ± 1.53 to 16.17 ± 2.06 µM, P = 0.036) and not in preterm (13.46 ± 2.48 to 13.65 ± 1.98 µM, P = 0.842) adults. However, no difference in Δ[deoxy(Hb+Mb)] at peak exercise intensity was observed between the groups in both normoxia and hypoxia. Hypoxia decreased cerebral O_2 saturation in term born but not in preterm adults at rest and during exercise (P < 0.05). As illustrated in **Figure 47**, $\dot{Q}O_2$ was lower in preterm compared to term born (5323 ± 389 vs. 6389 ± 271 mL·min^{-1}, P = 0.040) adults in normoxia, but not in hypoxia (4996 ± 412 vs. 4732 ± 226 mL·min^{-1}, P = 0.960). DO_2 was decreased by hypoxia to a similar extent in both groups (preterm: -21.2 ± 16.3 %·mL^{-1}·min^{-1}; term born: -22.4 ± 12.9 %·mL^{-1}·min^{-1}, P = 0.827).

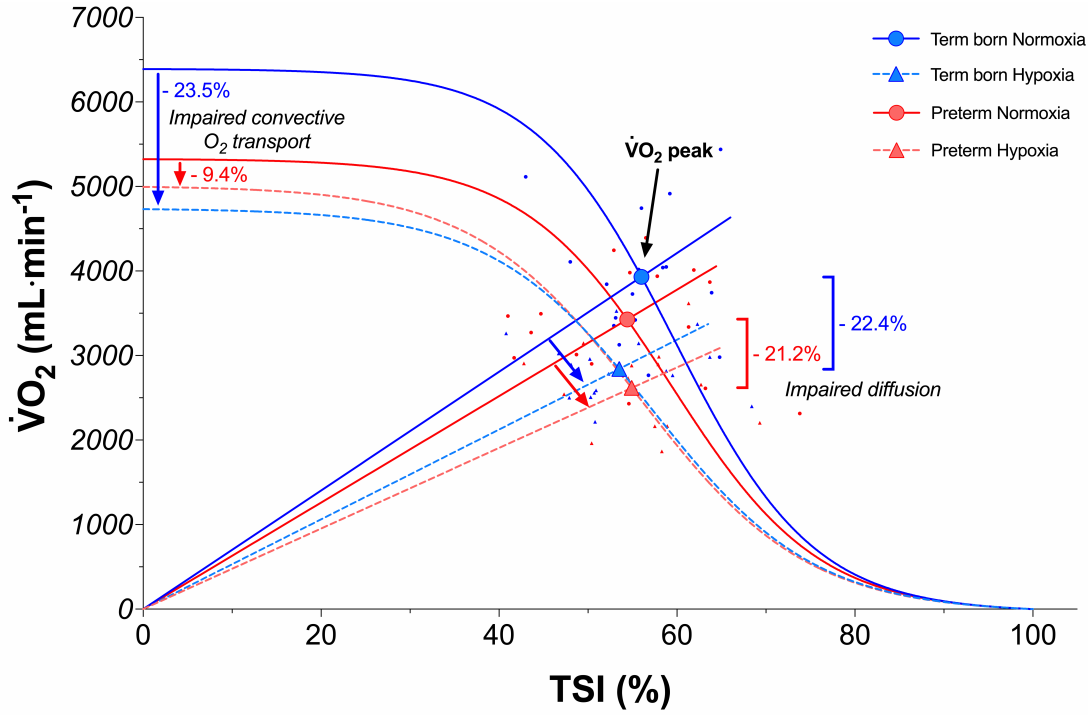

Figure 47. Graphical representation of the relative contribution of convective (sigmoid lines) and diffusive (straight lines from the origin) components of O_2 transport in prematurely born (in red) and term born (in blue) adults at sea-level (solid lines) and high-altitude (3375m, dotted lines) that integrate to determine $\dot{V}O_{2max}$. Convection can be mathematically described by the Fick Principle of Mass Conservation, while O_2 diffusion by the Fick's Law of diffusion. The intersection between these two relationships represents $\dot{V}O_{2max}$. See *Chapter 9, article 1* for further details. Individual normoxic and hypoxic $\dot{V}O_{2max}$ values are plotted for each group. TSI refers to *vastus lateralis* muscle Tissue Saturation Index measured by near infrared spectroscopy.

For more details, *see Chapter 9*, article 1.

5.6 Oxidative stress kinetic differs between preterm and term born adults during acclimatization to high-altitude

SOD did not change in term born adults, whereas it increased on both day 1 (+24%; $P = 0.003$) and day 3 (+12%; $P = 0.025$) compared to normoxia among those born preterm. Catalase activity did not change across the exposure in the control group. A transient increase was however observed upon arrival at altitude, relative to normoxia, in the preterm group (+58%; $P < 0.001$). Compared to normoxia, GPx increased from arrival at altitude in both groups (term

born: +7%, $P = 0.001$; preterm: +12%, $P < 0.001$), while FRAP decreased in both the control group (-15%; $P < 0.001$) and the preterm group (-8%; $P = 0.008$) at day 1. Moreover, XO increased upon arrival at altitude in term born participants (+124%, $P = 0.003$), and remained elevated relative to normoxia until day 3 of exposure. In contrast, in the preterm group, XO increased only at day 3 compared to normoxia (+65%, $P = 0.024$). MPO increased in both groups across the three days of exposure to high-altitude (term born: +31% at day 3 vs. normoxia, $P = 0.005$; preterm: +27% at day 3 vs. normoxia, $P = 0.004$). AOPP decreased at arrival in term born adults (-25%, $P = 0.022$), and remained reduced compared to normoxia until day 2 at high-altitude (-11.4%, $P = 0.028$). In the preterm cohort, AOPP increased, compared to normoxia, only at arrival (+28%, $P = 0.044$). MDA decreased in term born participants compared to normoxic values from arrival to day 3 at high-altitude (-17%, $P < 0.001$), while, among preterm adults, MDA decreased at arrival and returned (and remained stable) to baseline normoxic values from day 1 to day 3. NO_2^- increased on the third day at high-altitude (+42%, $P = 0.017$) compared to day 1 in term born participants, while a decrease at day 1 (-41%, $P = 0.009$) was observed in the preterm cohort compared to normoxia.

For more details, *see Chapter 13*, article 5.

6. Discussion and perspectives

Discussion

Premature birth was recently included in the list of chronic diseases, as growing evidence demonstrates persistent ventilatory, cardiorespiratory, cardiovascular and oxidative stress-related disorders during adulthood in this population. In turn, as young adults, these individuals present an increased risk for respiratory and cardiovascular diseases.

Exposure to hypoxia, whether simulated in a chamber (normobaric hypoxia) or during ascent to terrestrial altitude (hypobaric hypoxia), requires the body to implement several responses aiming to mitigate the negative consequences of the drop in O_2 availability. Although these responses are systemic, the respiratory and cardiovascular systems are among the firsts to respond to hypoxia. Giving the well-known alterations in ventilatory and cardiovascular functions associated with premature birth, it is of substantial clinical and physiological interest to understand how these individuals cope with decrease O_2 availability in ambient air.

This research project was the first to investigate in such a comprehensive way the respiratory, cardio- and cerebro-vascular, muscular, and hematological responses to acute and prolonged exposure to hypoxia in prematurely but otherwise healthy adults and age-matched peers born at term. We demonstrated *a*) lung function and D_LCO are not impaired in preterm compared to term born adults, as demonstrated by normal individuals predicted values and similar between-groups indices; *b*) upon arrival at high-altitude, preterm participants exhibited reduced skeletal muscle microvascular reactivity and DmO_2, as well as larger increase in oxidative stress, compared to their term born peers; *c*) hypoxia, but not hypercapnia, decreases BRS in term born but not in preterm adults; *d*) cerebral vasodilation during hypoxic exposure is blunted in preterm compared to term born individuals, together with different hypercapnic and hypocapnic cerebrovascular reactivity to CO_2; *e*) in normoxia, exercise capacity in prematurely born adults is impaired by reduced $\dot{Q}O_2$ with no differences in DO_2. Interestingly, hypoxia significantly affected $\dot{Q}O_2$ to a larger extent in term born than in preterm adults, leading to a

similar exercise capacity under hypoxia conditions between the two groups; and f) preterm, compared to term born, adults exhibited different oxidative stress kinetics during prolonged exposure to hypoxia. While oxidative stress was higher in preterm, compared to term born, participants upon arrival at high-altitude, preterm individuals seemed to develop a better anti-oxidant defense system over the following days sojourning at high-altitude.

In these studies, we investigated extremely-to-very (range gestational age, 26 – 32 weeks) prematurely born adults who underwent O_2 supplementation therapy immediately after birth. Although detailed information on the protocol and duration of the postnatal O_2 treatment in our preterm cohort is missing, previous evidence suggested an association between neonatal exposure to elevate O_2 concentration and both lung damage (Lignelli *et al.*, 2019; Gibbons *et al.*, 2020) and chemoreceptors dysfunction (Bates *et al.*, 2014) during adulthood. However, our preterm cohort was screened for absence of BPD, and demonstrated normal lung function and D_LCO, as well as similar $\dot{V}_E$ at the point of volitional exhaustion, compared to their term born and age-matched counterparts. Data on impaired resting pulmonary function and D_LCO are contradictory, with either reduced (Vrijlandt *et al.*, 2006; Duke *et al.*, 2014) or normal (Farrell *et al.*, 2015; Debevec *et al.*, 2019) lung function in healthy premature adults compared to term born peers. Our data suggest that healthy and moderately active prematurely born adults may not present impaired resting pulmonary function and diffusion capacity. This is in line with a recent work reporting minor lung function abnormalities in 11-year-old children born preterm in the turn of the millennium compared to those born in the early 1990s (Vollsaeter *et al.*, 2015), suggesting that a re-examination of the currently accepted paradigm of deleterious long-term respiratory consequences induced by premature birth is necessary.

Growing evidence also described a specific cardiovascular phenotype at rest in adults born prematurely, both in normoxia (Goss *et al.*, 2020; Harris *et al.*, 2020; Schuermans & Lewandowski, 2022) and hypoxia (Barton *et al.*, 2021), which ultimately increases the risk for

adverse cardiovascular events in these individuals. These observations are however primarily based on findings at cardiac level and/or major artery vessels. Considering that microcirculatory dysfunction has been identified as early marker for later cardiovascular diseases, it is surprising that only very little attention has been given to microcirculation. Impaired cutaneous microcirculatory function in preterm neonates (Puchwein-Schwepcke *et al.*, 2019) and altered pulmonary microvascular hemodynamics, as well as microvascular function and density, in adults born preterm with normal lung function (Barton *et al.*, 2020; Lewandowski *et al.*, 2020) were recently described. In the present work, microvascular function, assessed in terms of post-occlusive NIRS-derived microvascular reactivity, was similar between prematurely born and term born participants in normoxia. However, upon acute exposure to hypoxia, reperfusion rate increased and time to return to baseline muscle oxygenation (i.e., $t_{baseline}$) became faster in term born, but not in preterm, adults, suggesting a blunted vasodilatory capacity in response to hypoxia in the latter group. Indeed, as extensively reviewed in the second chapter of this thesis, hypoxia triggers vasodilation at skeletal muscle via release of pro-angiogenic factors and local vasodilators (i.e., NO, adenosine, acidosis), leading to increased microcirculatory oxygen extraction capacity (Mirna *et al.*, 2020). Interestingly, the hyperemic area (i.e., AUC_{2MIN}), despite being lower in preterm born adults in both conditions, was similarly affected by hypoxia in both groups. Alterations in vascular function within the microcirculation were recently proposed to precede and predict conduit artery dysfunction (Gutterman *et al.*, 2016). At this regard, cerebral vasodilation in the middle cerebral artery during hypoxic exposure was also blunted in preterm adults compared to their peers born at term, further reinforcing the notion that some endothelium-dependent or -independent mechanisms underlying vessel dilation might be impaired in the former adult cohort. However, further work is required to confirm the mechanism(s) underlying this reduced vasodilatory response to hypoxia, and its clinical significance, in prematurely born adults.

Ultimately, these impaired micro- and macro-vascular responses to hypoxia may mechanistically underpin the potentially increased susceptibility of these individuals to high-altitude illnesses, in particular to high-altitude pulmonary and cerebral edema (Debevec *et al.*, 2022a).

At a more central level, Barton and colleagues recently reported an exaggerated cardiac contractile response, especially at the level of the RV, in healthy preterm adults acutely exposed to hypoxia, despite similar increase in mean pulmonary artery pressure (Barton *et al.*, 2021). This hyper-adaptive response of the RV to hypoxia was also reported in a previous animal model using postnatal hyperoxia to simulate premature birth in humans (Goss *et al.*, 2015). Although the mechanisms underlying this greater increase in RV contractility in hypoxia in the preterm heart remain to be elucidated, it appears that the preterm RV is hyper-adaptive to a pressure challenge (such us hypoxia), and that perturbation in autonomic nervous system function may underlie this abnormal response. Indeed, recent work investigating autonomic function post-exercise reported alteration in cardiac autonomic control in both in adolescents (Haraldsdottir *et al.*, 2018a) and young adults born preterm (Haraldsdottir *et al.*, 2019). Although mechanistic evidence is still missing, some authors speculated these autonomic perturbations in cardiovascular control to be a consequence of neonatal O_2 supplementation therapy (Barton *et al.*, 2021). In the present thesis, we indirectly assessed the autonomic control of the cardiovascular system in terms of BRS (La Rovere *et al.*, 2008). Hypoxia decreased BRS among term born participants, while it did not change in the preterm cohort. Since hypoxia represent a small pressor challenge for the cardiovascular system, our data support previous speculations that the preterm cardiovascular system presents a hyper-adaptive phenotype to pressor changes (Barton *et al.*, 2021). Intriguingly, BRS was similar between term born and preterm participants while breathing hypercapnic gas mixtures either at sea-level or at high-altitude. The discrepancy between greater BRS in hypoxia and not in hypercapnia in preterm compared to term born adults might be due to a different exposure time to the breathing

stimulus. Indeed, BRS was assessed after about 20 h after arrival at high-altitude, while the hypercapnic breathing period was only 6 minutes.

A central debate in prematurity-related research is about oxidative stress modulation in this adult population. It is well-known that neonatal exposure to elevated O_2 concentrations represents a huge source of ROS overproduction (Clanton, 2007), and previous work demonstrated greater oxidative stress at rest in normoxia in both newborns and adults born prematurely (Menon, 2014; Martin *et al.*, 2018). In turn, the subsequent redox imbalance might result in a 'preconditioning' state, or it may be involved in the pathogenesis of several non-communicable chronic cardiovascular and metabolic diseases (Sutherland *et al.*, 2014; Pena-Oyarzun *et al.*, 2018; Flahault *et al.*, 2020b; Deprez *et al.*, 2021). Although we did not observe higher oxidative stress in normoxia in our preterm cohort compared to their peers born at term, hypoxia is known to increase ROS production and lead to redox imbalance (Clanton, 2007). Recent results from our research group showed a greater resistance to hypoxia-induced oxidative stress in preterm compared to term born adults (Debevec *et al.*, 2019; Martin *et al.*, 2020), suggesting a status of "hypoxic precondition", in terms of pro-oxidant and anti-oxidant balance, in the former group of individuals. However, these studies were conducted in acute normobaric hypoxic exposure (few minutes) and therefore a complete understanding of the potential role of oxidative stress modulation on hypoxic acclimatization in this population was still missing. Our current data demonstrated that preterm adults, compared to term born peers, exhibit larger increases in oxidative stress upon exposure to hypobaric hypoxia (15 minutes after arrival at high-altitude), although a better redox balance was observed in the former cohort over the following days sojourning in at high-altitude. It seems therefore that, despite an initial "peak" in oxidative stress and in contrast with cardio- and cerebrovascular responses, adults born preterm may have developed molecular adaptative mechanisms to hypoxia aiming to cope with large variations in oxygen experienced at birth and known to increase the ROS production.

Likewise, we also observed a lower altitude-induced decrease in oxidative capacity in our preterm cohort compared to their term born peers upon exposure to hypobaric hypoxia. These data, although being indirect, provide further support on a specific phenotypical response, or better "non-response", to hypoxia in the preterm population. However, oxidative function at the skeletal muscle level remains significantly under-investigated in this population, with current evidence reporting either elevated mitochondrial oxygen consumption in peripheral blood mononuclear cells of prematurely born adults (Kumari *et al.*, 2021) or reduced mitochondrial function in male rats exposed to postnatal hyperoxia (Tetri *et al.*, 2018). Several comorbidities typically described in adult survivors of premature birth are characterized by larger mitochondrial dysfunction and chronic oxidative stress than in non-preterm population. Further investigation of the longitudinal effects of premature birth on mitochondrial function, and the potential modulation induced by hypoxia, may help to further understand the mechanisms underlying the increased risk for several diseases in this population.

Lastly, it is widely described in the literature that there is a lower exercise capacity in normoxia in preterm adults compared to age-matched peers born at term (Vrijlandt *et al.*, 2006; Lovering *et al.*, 2013; Duke *et al.*, 2014; Farrell *et al.*, 2015; Haraldsdottir *et al.*, 2018b; Debevec *et al.*, 2019). When preterm participants with BPD are excluded, limitations in cardiac size and/or dimensions seems to be the primary mechanisms by which exercise capacity is reduced in healthy preterm adolescents and young adults (Haraldsdottir *et al.*, 2018b; Huckstep *et al.*, 2018; Haraldsdottir *et al.*, 2020; Huckstep *et al.*, 2021; McKay *et al.*, 2021). Especially, in agreement with the previous investigations (Huckstep *et al.*, 2018; Haraldsdottir *et al.*, 2020; Huckstep *et al.*, 2021), we provided further evidence of a very small stroke volume reserve in exercising preterm, compared to term born individuals. However, the specific mechanism behind the smaller stroke volume reserve in this cohort remain debated. During my stay at the University of Texas Southwestern Medical Center we sought to answer this question by

performing cardiac MRI scans during supine exercise at increasing intensity in preterm and term born adults with normal resting lung function and LV ejection fraction. Although data analysis is still ongoing (and therefore I was unable to include those findings in the present thesis), preliminary results suggest lower biventricular end-diastolic volumes with increasing exercise intensity in preterm compared to term born adults. Whether this altered cardiac mechanics during exercise is secondary to reduced ventricular filling dynamics remains to be clarified. Intriguingly, despite altitude exposure reduced peak power output to a similar extent in both groups, the hypoxia-induced decrease in cardiac hemodynamics at maximal exercise intensity was significantly lower in preterm adults compared to their term born peers. Indeed, term born participants exhibited a two-fold greater drop in $\dot{Q}O_2$ at peak exercise intensity from normoxia to hypoxia compared to the preterm cohort (-23.5% vs. -9.4%). Our results are in line with previous findings in preterm humans, where hypoxia mitigated the lower power output observed in preterm individuals during normoxic exercise (Farrell *et al.*, 2015), as well as in a rodent model simulating premature birth by postnatal hyperoxic exposure (Goss *et al.*, 2015). In this latter study, preterm adult rats showed a better maintenance of $\dot{Q}$ and exercise performance ($\dot{V}O_{2peak}$) after hypoxia compared control subjects exposed to room air postnatally (Goss *et al.*, 2015). These results led the authors to raise an important question on whether "*cardiac and possibly skeletal muscle responses to preterm births condition those individuals, in some ways, to be primed for a more resilient response to later hypoxic events*" (Goss *et al.*, 2015). Thanks to this doctoral project we have now more data (**Figure 48**) to answer this important point. At rest, prematurely born adults exposed to hypoxia seem to not present this "hypoxic preconditioning" state, as originally hypothesized. Especially, at cardio- and cerebro-vascular levels, the blunted vasodilatory responses and exaggerated cardiac contractility to hypoxia may predispose these individuals to an increased risk for cardiovascular events at altitude and altitude-related disorders.

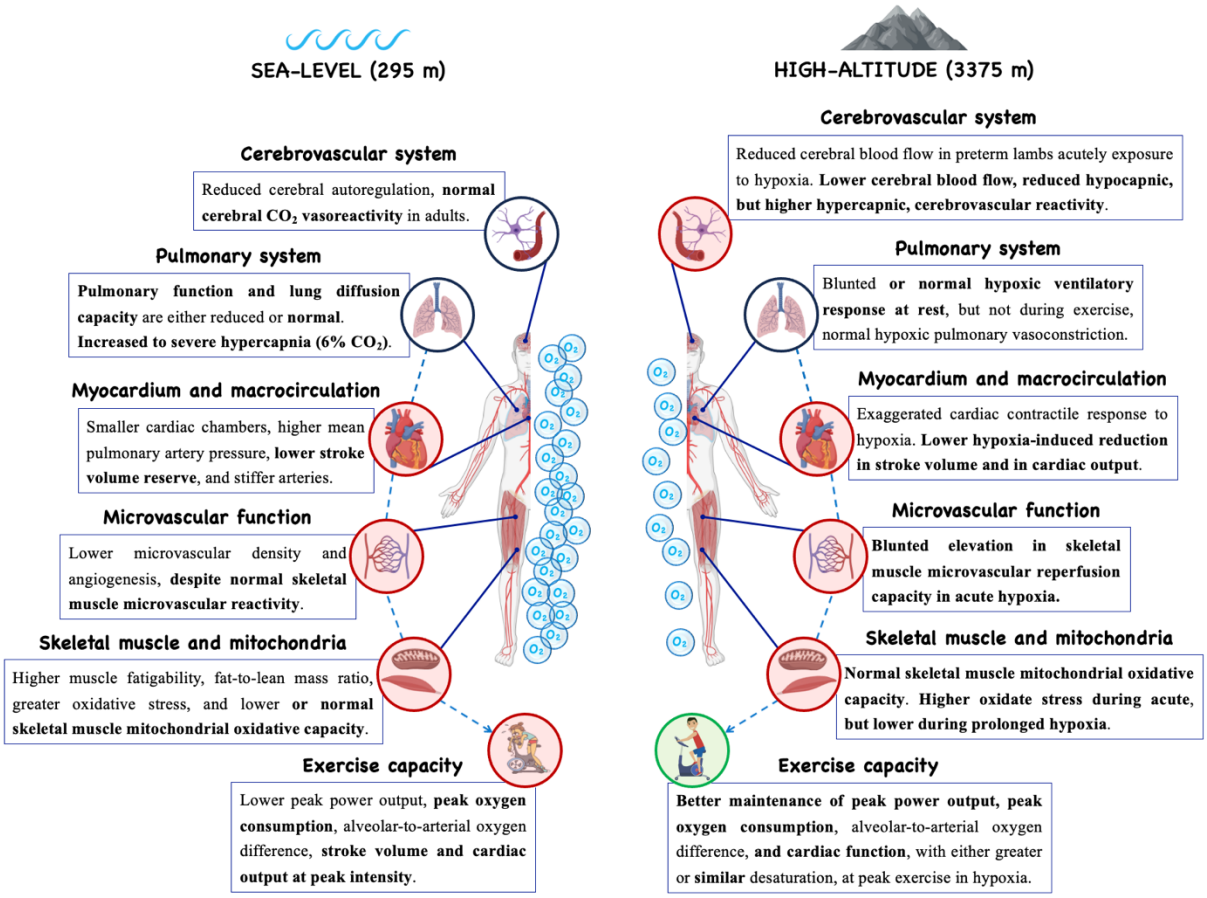

Figure 48. Updated summary of the existing findings on the physiological responses at rest and during exercise in normoxia and hypoxia in prematurely born individuals compared to their peers born at term. In bold, the findings from the present doctoral project are highlighted. In red, the altered physiological functions described in preterm individuals compared to term born peers; in white those functions on which contrasting findings are currently available; and in green, those responses that are beneficial in preterm vs. term born individuals. The light blue dotted lines and arrows highlights the systems involved in the O_2 cascade and that ultimately determine exercise capacity.

However, as shown in **Figure 48** that summarizes the main findings of the present doctoral thesis, this "hypoxic preconditioning" effect is unmasked by exercise, in line with previous works of a better maintenance of exercise performance (Farrell *et al.*, 2015), $\dot{Q}$ (Goss *et al.*, 2015), and HVR (Debevec *et al.*, 2019) in this cohort compared to their term born peers. At this point, it appears clear that the "hypoxic preconditioning" paradigm in preterm adults is too simplistic since it does not consider the complex and multiple interactions between several systems (cerebrovascular, pulmonary, myocardium and macrocirculation, microvascular function, and skeletal muscle and mitochondria) (**Figure 48**).

Perspectives

Global premature birth rates have increased considerably across the last three decades (Liu *et al.*, 2016), and concomitant improvements in neonatal intensive care (thus lower mortality rates) led to an ever-increasing number of preterm birth survivors reaching adulthood. However, research exploring the lasting effects of preterm birth beyond maturity and the potential effects of stressors, such as exercise or exposure to hypoxia, is still relatively new and limited.

As extensively discussed in the previous chapters, exercise capacity is typically reduced in preterm compared to term born participants, and the measured $\dot{V}O_{2peak}$ of these individuals is often at, or just slightly above, the threshold for very poor aerobic fitness. However, our preterm cohort demonstrated a relatively high $\dot{V}O_{2peak}$ compared to previous values in the literature (Vrijlandt *et al.*, 2006; Duke *et al.*, 2014). It might be speculated that an active childhood and adolescence may help to contrast the prematurity-associated decline in cardiorespiratory fitness in adult age. This hypothesis find support in recent findings of a specific training window during adolescence that allow to gather greater benefits of exercise training which persist during adulthood (Perkins *et al.*, 2022).

Furthermore, NO regulates vascular tone and blood flow, primarily by eliciting vasodilation (Casey *et al.*, 2010), and insufficient NO bioavailability seems to be one of the mechanisms underlying maladaptation to high-altitude (Bailey *et al.*, 2010). Early findings in rodent models also suggest that elevated blood ketones may lower lactate levels at cerebral level, suggesting decreased glycolysis or increased glucose disposal, leading to an overall greater tolerance to hypoxia (Kirsch *et al.*, 1980; Puchowicz *et al.*, 2005). These results were recently extended in humans, where, despite no beneficial effects on performance, greater blood and muscular (and potentially also cerebral) oxygenation were observed during exercise in hypoxia following ketone supplementation in highly trained males (Poffe *et al.*, 2021).

Future studies should therefore investigate the efficacy of exercise training and lifestyle intervention (i.e., NO and ketones supplementation) in children and adolescents born prematurely, and the lasting effects of these physical activity programs on exercise capacity and cardiorespiratory fitness during adulthood.

A second point of discussion in terms of future research is about sex differences. Preliminary data in animals indicated sex differences in the physiological responses to both rest and exercise in preterm adults (Tetri *et al.*, 2018; Goss *et al.*, 2020), with greater responses and lower risk for cardiometabolic disorders in females born preterm compared to their male peers (Shim *et al.*, 2017). However, the mechanisms underlying these differences between preterm males vs. females remain unknown. It is also known that females present specific responses to hypoxia, also depending on the phase of the menstrual cycle (Richalet *et al.*, 2020). Further work is therefore required to understand the factors regulating sex differences in the physiological response to rest, exercise, and hypoxia in prematurely born adults.

Lastly, existing evidence on the responses to hypoxia in preterm adults is drawn on lowlander participants only. It might be of great interest to compare the physiological changes induced by exposure to hypoxia in lowlander vs. highlander preterm born adults. Especially, the long-term genetic adaptations to hypoxia typically described in highlander populations may mitigate the blunted vascular response observed in the present cohort of preterm individuals.

7. Conclusion

General conclusion

The aim of the present doctoral thesis was to investigate the physiological responses to hypoxia in term born and prematurely but otherwise healthy adults. Especially, we sought to elucidate the differences in respiratory, cardiovascular, cerebrovascular, and muscular function, as well as in oxidative stress responses, during acute and prolonged exposure to hypobaric hypoxia between term born vs. preterm born individuals. Our findings can be summarized as *a*) lung function and the ventilatory response to both hypoxia and hypercapnia are similar in preterm and term born adults; *b*) vasodilatory capacity in response to hypoxia is reduced both at skeletal muscle microvascular level and in major cerebral artery vessels; *c*) oxidative stress kinetics during acute and prolonged high-altitude exposure differs in preterm vs. term born adults, with larger increases in acute, but not during prolonged, exposure in the formed group; and *d*) although impaired stroke volume reserve underlie the reduced exercise capacity in preterm adults in normoxic condition, these adults present a lower susceptibility to the detrimental effects of hypoxia on exercise capacity and cardiac performance than their term born peers.

In conclusion, our findings suggest that individuals born prematurely do no present a "hypoxic preconditioning" effect at rest, as it was previously hypothesized. The blunted vasodilatory capacity unmasked by the use of hypoxia is a critical finding which would require further investigation to understand the underlying mechanisms. The use of physiological stressors such as exercise and/or hypoxia seems therefore central in delineating functional differences between adults with or without a history of premature birth.

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9. Article 1

Physiological responses to exercise in hypoxia in preterm adults: convective and diffusive limitations in the O_2 transport

Article 1 - Physiological responses to exercise in hypoxia in preterm adults: convective and diffusive limitations in the O_2 transport

Manferdelli G, Narang BJ, Bourdillon N, Debevec T & Millet GP. (2023). *Med Sci Sports Exerc* **55,** 482-496.

Physiological Responses to Exercise in Hypoxia in Preterm Adults: Convective and Diffusive Limitations in the O_2 Transport

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BASIC SCIENCES

ABSTRACT

MANFERDELLI, G., B. J. NARANG, N. BOURDILLON, T. DEBEVEC, and G. P. MILLET. Physiological Responses to Exercise in Hypoxia in Preterm Adults: Convective and Diffusive Limitations in the O_2 Transport. *Med. Sci. Sports Exerc.*, Vol. 55, No. 3, pp. 482–496, 2023. **Purpose:** Premature birth induces long-term sequelae on the cardiopulmonary system, leading to reduced exercise capacity. However, the mechanisms of this functional impairment during incremental exercise remain unclear. Also, a blunted hypoxic ventilatory response was found in preterm adults, suggesting an increased risk for adverse effects of hypoxia in this population. This study aimed to investigate the oxygen cascade during incremental exercise to exhaustion in both normoxia and hypobaric hypoxia in prematurely born adults with normal lung function and their term born counterparts. **Methods:** Noninvasive measures of gas exchange, cardiac hemodynamics, and both muscle and cerebral oxygenation were continuously performed using metabolic cart, transthoracic impedance, and near-infrared spectroscopy, respectively, during an incremental exercise test to exhaustion performed at sea level and after 3 d of high-altitude exposure in healthy preterm (n = 17; gestational age, 29 ± 1 wk; normal lung function) and term born (n = 17) adults. **Results:** At peak, power output, oxygen uptake, stroke volume indexed for body surface area, and cardiac output were lower in preterm compared with term born in normoxia (P = 0.042, P = 0.027, P = 0.030, and P = 0.018, respectively) but not in hypoxia, whereas pulmonary ventilation, peripheral oxygen saturation, and muscle and cerebral oxygenation were similar between groups. These later parameters were modified by hypoxia (P < 0.001). Hypoxia increased muscle oxygen extraction at submaximal and maximal intensity in term born (P < 0.05) but not in preterm participants. Hypoxia decreased cerebral oxygen saturation in term born but not in preterm adults at rest and during exercise (P < 0.05). Convective oxygen delivery was decreased by hypoxia in term born (P < 0.001) but not preterm adults, whereas diffusive oxygen transport decreased similarly in both groups (P < 0.001 and P < 0.001, respectively). **Conclusions:** These results suggest that exercise capacity in preterm is primarily reduced by impaired convective, rather than diffusive, oxygen transport. Moreover, healthy preterm adults may experience blunted hypoxia-induced impairments during maximal exercise compared with their term counterparts. **Key Words:** PREMATURE BIRTH, ALTITUDE, CARDIORESPIRATORY, NIRS, EXERCISE CAPACITY

Premature birth (<37 wk of gestation) affects over 10% of all live births worldwide, and those born "very" preterm (≤32 wk of gestation) often exhibit significant respiratory, pulmonary, and cardiovascular limitations that may persist into adulthood (1–3). Growing evidence supports long-term consequences (i.e., hypertension, impaired respiratory control, metabolic and cardiovascular diseases) in young adults born prematurely (2,4,5).

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Recent reviews on the topic highlighted cardiac and hypertensive pulmonary vascular remodeling as leading mechanisms for the increased risk of cardiovascular events and mortality in prematurely born individuals (1,5). In addition, despite similar physical activity levels, these alterations are related to lower exercise capacity in normoxia (6) in preterm adolescents (7) and adults (8–10). Despite suggestions that cardiopulmonary dysanapsis might chiefly underpin the observed prematurity-related exercise intolerance (1), the exact causes remain unclear.

Hypoxia is known to significantly impair maximal exercise capacity (11). However, the evidence on the relationship between the individual resting hypoxic ventilatory response (HVR) and the hypoxia-induced decrease in exercise capacity remains equivocal (12,13). Preterm individuals have a blunted HVR at rest (10,14). Despite these latter studies investigating HVR in healthy preterm adults with normal lung function and exercise capacity, rodent models and human studies suggest that perinatal oxygen (O_2) therapy likely impairs both peripheral and central components of the ventilatory drive (14,15).

Importantly, not all preterm newborns undergo O_2 therapy, and this needs to be considered when interpreting existing literature. However, the long-term impact of premature birth on high-altitude adaptation capacity and/or on resting and exercise physiological responses during exposure to hypoxia is still uncertain. Few studies showed reduced (10) or unchanged (8) maximal power output in hypoxia in this population compared with term born peers, as well as no clear influence of premature birth on gas exchange efficiency (9) during exercise in hypoxia. Similarly, an animal model aiming to simulate premature birth in humans showed a greater maintenance of cardiac output and peak oxygen consumption ($\dot{V}O_{2peak}$) in hypoxia in adult rats exposed to neonatal O_2 supplementation, compared with those who breathed room air postnatally (16).

Because exercise capacity is determined by the integrative response of multiple systems (i.e., cerebrovascular, respiratory, cardiovascular, and musculoskeletal systems), this study aimed to investigate the full oxygen cascade during incremental exercise to exhaustion in both normoxia and hypobaric hypoxia in prematurely born but otherwise healthy adults and term born age-matched individuals. We measured gas exchange, cardiac function, and muscle and cerebral oxygenation, at rest, across submaximal work rates, and at peak exercise. Moreover, because maximal oxygen consumption ($\dot{V}O_{2max}$) is determined by both convective ($\dot{Q}O_2$) and diffusive (DO_2) components of O_2 transport, we used the model proposed by Wagner (17) to investigate these two integrated components. We hypothesized lower exercise capacity and $\dot{V}O_{2peak}$ in adults born preterm compared with term born peers because of impairments at the convective rather than diffusive O_2 transport level. Moreover, we expected these differences to be exacerbated in hypoxia.

METHODS

Participants and Ethical Approval

Thirty-four young healthy men volunteered and gave written informed consent to participate in this study. Seventeen participants were born preterm, and 17 were born at term. The preterm born participants were recruited via the National preterm birth register managed by the University Clinical Centre in Ljubljana, Slovenia, using medical record screening and telephone/email-based individual interviews. The inclusion criteria for them were gestational age ≤32 wk, birth weight ≤1500 g, O_2 therapy at birth, and absence of bronchopulmonary dysplasia diagnosis. The inclusion criteria for the term born participants were gestational age ≥38 wk and birth weight ≥2500 g. Birth-related inclusion criteria for all participants were checked and confirmed during the initial birth/medical record screening procedure conducted before inclusion in the study. Exclusion criteria for all participants comprised permanent altitude residence (≥1000 m), cardiopulmonary, hematological and/or kidney disorders, chronic medication use, smoking, and altitude/hypoxia exposure (≥2000 m) within the last month before the study. Participants were matched for age, height, and body mass. This study was performed according to the Declaration of Helsinki. The experimental protocol was preregistered at ClinicalTrials.gov (NCT04739904), and ethical approvals were obtained from both the University of Ljubljana, Faculty of Sport ethics committee (8/2020-316), and the Aosta Hospital Ethical Committee (06/05/2021.0038781.I).

Experimental Design and Ascent Profile

Each participant underwent two experimental trials (outlined below), one near sea level (295 m; Ljubljana, Slovenia; barometric pressure ~737 ± 2 mm Hg) and the other after 3 d sojourning at high altitude (3375 m; Torino hut, Aosta Valley, Italy; barometric pressure ~503 ± 3 mm Hg). Participants traveled from Ljubljana to Courmayeur (1300 m) by car, before reaching the Torino hut by cable car. The cable car journey lasted approximately 20 min. To avoid the potential influence of high-altitude illness, symptoms of acute mountain sickness were assessed in the morning upon awake using the Lake Louise Scale (18).

Experimental Protocol

Before each experimental session, the participants were instructed to abstain from heavy exercise and avoid alcohol and caffeine for 24 h. They did not consume a heavy meal within the 4-h prior testing. Participants performed an acute transient hypoxic chemosensitivity protocol, spirometry, and an incremental exercise test to exhaustion at sea level, and repeated the latter test at high altitude. All exercise tests were conducted using an electrically braked cycle ergometer (Premium 8i ergo bike; Daum Electronic gmbh, Fürth, Germany). After 5 min of seated rest, participants began cycling at 60 W for 2 min, followed by increments of 40 W every 2 min until they could no longer maintain the required cadence despite strong verbal encouragement. Throughout the tests, participants were instructed to maintain a pedaling frequency between 75 and 85 rpm. Maximal exhaustion was considered attained when at least three of the following parameters were observed: capillary lactate concentration ($[La]_b$) >8 mmol·L^{-1} within 1 min after exercise cessation, a respiratory exchange ratio >1.10, a maximal heart rate (HR) >85% of the participant's predicted maximal HR, or a rating of perceived exertion higher than 18 using the Borg's 6–20 scale (19).

Measurements

Acute transient hypoxic chemosensitivity. After a 3-min baseline period, participants were exposed to 10 periods of 100% nitrogen inhalation (20). Each period consisted of 1–8 consecutive breaths in a randomized order aiming to induce a wide range of arterial oxygen desaturations (~99% to 54%). Transitions from ambient normoxic air to full nitrogen breathing were accomplished using a three-way T-shaped valve (Hans Rudolph, 2100 series; Hans Rudolph, Kansas City, MO) in a participant-blinded manner. Consecutive

nitrogen exposures were separated by ~2 min or until respiratory parameters returned to baseline values. Pulmonary ventilation ($\dot{V}_E$) and peripheral oxygen saturation (SpO_2) were continuously monitored during the test by a metabolic cart (Ergocard Professional; Medisoft, Sorinnes, Belgium) and a pulse oximeter (Xpod 3012LP; Nonin Medical Inc, Plymouth, MN) placed on the ear lobe. The lowest measured SpO_2 value measured within the 30 s after each period of 100% nitrogen inhalation was plotted against the corresponding peak in $\dot{V}_E$.

Pulmonary function. Forced vital capacity (FVC), forced expired volume in 1 s (FEV_1), FEV_1/FVC ratio, peak expiratory flow, and maximal voluntary ventilation (MVV) were obtained before the incremental exercise test using a portable spirometer (Cardiovit AT-2plus; Schiller, Baar, Switzerland), according to standardized procedures (21). Single-breath lung diffusion capacity for carbon monoxide (DL_{CO}) and alveolar volume were assessed in normoxia (Vyntus™ ONE Pulmonary Function System; Vyaire Medical Inc., Mettawa, IL) according to standard procedures (22). A venous blood sample was taken from the antecubital vein of each participant, and DL_{CO} values were adjusted for individual hemoglobin (Hb) concentration (22). Lung function and DL_{CO} parameters were expressed in both absolute terms and as a percentage of predicted values using established equations (23,24). DL_{CO} measurement from two preterm and one term born participants were not available. Overall, the presented DL_{CO} data include $n = 16$ prematurely born and $n = 15$ term born adults.

Pulmonary gas exchange. Respiratory parameters, including oxygen uptake ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), $\dot{V}_E$, breath frequency, tidal volume (V_T), and end-tidal partial pressure of carbon dioxide ($P_{ET}CO_2$), were monitored continuously, breath by breath, using a computerized metabolic cart (Ergocard Professional; Medisoft). Before each experiment, calibration of the device was performed with a 3-L syringe at two different flow rates following the manufacturer's instruction, whereas O_2 and CO_2 analyzers were calibrated using gas mixtures of known concentration. Gas exchange threshold (GET) was identified using the V-slope analysis of $\dot{V}CO_2$ plotted against $\dot{V}O_2$, and it was confirmed by specific trends of $\dot{V}_E$ versus $\dot{V}O_2$ ($\dot{V}_E/\dot{V}O_2$) and $\dot{V}CO_2$ ($\dot{V}_E/\dot{V}CO_2$), as well as of both $P_{ET}O_2$ and $P_{ET}CO_2$ (25,26). Similarly, the respiratory compensation point (RCP) was independently identified by two physiologists as the point of increase in the $\dot{V}_E/\dot{V}CO_2$ relationship, and confirmed by a simultaneous reduction in $P_{ET}CO_2$ (26). In the event of a disagreement between the two physiologists, a third physiologist performed the evaluation and the average of the two closest assessments was picked as RCP. $\dot{V}O_2$ corresponding to both GET and RCP was identified by a linear $\dot{V}O_2$ versus time relationship. Peak values were calculated as the highest value averaged over the last 20 s of exercise, whereas peak power output was calculated as the highest power output recorded immediately before exercise cessation. Breathing reserve, an index of ventilatory limitation to exercise (27), was calculated as ($\dot{V}_E$/MVV) × 100. The work of breathing (Wb) and the $\dot{V}O_2$ used by the respiratory muscles ($VRMO_2$) were computed from $\dot{V}_E$ using the equations proposed by Coast et al. (28):

$$Wb = -0.251 + 0.0382 \times \dot{V}_E + 0.00176 \times \dot{V}_E^2 \quad [1]$$

$$VMRO_2 = 34.9 + 7.45 \times Wb \quad [2]$$

Exercise-induced hypoxemia. The occurrence of exercise-induced hypoxemia (EIH) was determined during the normoxic incremental test by continuously monitoring SpO_2 by pulse oximeter (Xpod 3012LP; Nonin Medical Inc) at the ear lobe. EIH was defined as a drop ≥4% from preexercise SpO_2 for at least 3 consecutive minutes (29).

Hemodynamic responses. HR and stroke volume normalized for body surface area (SVi) were noninvasively measured beat-by-beat by transthoracic impedance (Physioflow Enduro; Manatec Biomedical, Paris, France) and averaged every 1 s. The accuracy of this device in monitoring cardiodynamics during incremental exercise in healthy individuals was previously evaluated against the direct Fick method (30), and the device was previously used at altitude (31). Absolute cardiac output ($\dot{Q}$) and cardiac output normalized to body surface area ($\dot{Q}i$) were calculated as HR × SV and HR × SVi, respectively. To optimize the impedance signal, skin was carefully shaved and cleaned, and electrodes were secured on the skin using tape. Blood pressure was measured on the left arm at rest before each test using a digital blood pressure monitor (M2; OMRON Healthcare, Hoofddorp, the Netherlands). HR, SVi, and $\dot{Q}i$ at rest and peak exercise were calculated as the average over the last 5 s before each time point. Data points with a signal quality less than 80% were excluded from analyses.

Muscular and cerebral oxygenation. Oxygenation changes in the vastus lateralis muscle and in the prefrontal cortex were evaluated using near-infrared spectroscopy (NIRS). For skeletal muscle, a portable NIR continuous-wave photometer (PortaMon; Artinis Medical Systems, Elst, the Netherlands) was utilized. The instrument measures concentration changes of oxygenated Hb and myoglobin (Mb) {Δ[oxy(Hb + Mb)]} and deoxygenated Hb and Mb {Δ[deoxy(Hb + Mb)]} with respect to an initial value arbitrarily set equal to 0 μM. The sum of these two variables {Δ[oxy(Hb + Mb) + deoxy(Hb + Mb)]} is related to changes in total blood volume in the muscle region of interest. Δ[Deoxy(Hb + Mb)] was considered to estimate skeletal muscle fractional O_2 extraction because this variable, contrarily to Δ[oxy(Hb + Mb)], is relatively insensitive to changes in blood volume (32). A "physiological calibration" of Δ[deoxy (Hb + Mb)] values was performed before the test by obtaining a maximal deoxygenation of the muscle. This was attained by pressure cuff inflation to 290–310 mm Hg at the root of the thigh (with the participants sitting on the cycle ergometer) for a few minutes until Δ[deoxy(Hb + Mb)] reached a plateau. The Δ[deoxy(Hb + Mb)] signal during ischemia was normalized for blood volume changes using the correction proposed by Ryan and colleagues (33). In addition, the muscle tissue O_2 saturation index (mTSI, %; i.e., ratio between Δ[oxy (Hb + Mb)] and Δ[oxy(Hb + Mb) + deoxy(Hb + Mb)]x100) is an absolute index commonly adopted to reflect tissue O_2

availability (32). The skin overlying the investigated muscle region was carefully shaved before experimentation, and the same experimenter placed the probe to minimize the variability in positioning between tests. The probe was secured to the skin using double-sided tape, and dark elastic bandages were wrapped around the probe and the skin to avoid any contamination from ambient light. Adipose tissue thickness at the site of application of the NIRS probe was measured by a skinfold caliper.

For cerebral tissue, brain TSI, and changes in the concentrations of deoxygenated (Δ[deoxyHb]), oxygenated (Δ[oxyHb]) and total hemoglobin (Δ[deoxyHb + oxyHb]) were monitored using a portable NIRS device (PortaLite; Artinis Medical Systems) placed in correspondence with the left prefrontal cortex. A headset held a NIR emitter (laser light at 780 and 850 nm) and three detectors over the left prefrontal cortex region of the forehead. Three distances (30, 35, and 40 mm) were adopted between the receiver and transmitters. Optodes were held in place using double-sided tape and covered by a black bandage. The sampling frequency for both muscle and brain NIRS was set at 10 Hz, and data were exported at 5 Hz for later analyses. Muscle and brain NIRS indices at rest and peak exercise were calculated as the average over the last 10 s before each time point.

Intramuscular matching between O_2 delivery and O_2 utilization. The presence of a transitory deoxygenation ([deoxy(Hb + Mb)]) "overshoot" was investigated in the transition from rest to exercise during the 2 min of exercise at 60 W (32,34). Data were averaged every 1 s and then fitted by a double exponential function of the type:

$$y(t) = y_{bas} + A_u\left[1 - e^{(t - TD_u)/\tau u}\right] + A_d\left[1 - e^{(t - TD_d)/\tau d}\right] \quad [3]$$

where y_{bas} indicates the baseline, A_u the amplitude of the upward component observed within the first 20 s of exercise, TD_u is the time delay, and τ_u is the time constant of the upward (u) component of the function. A_d, TD_d, and τ_d indicate the amplitude, time delay, and time constant of the downward (d) component, respectively. The amplitude (A) of the ([deoxy(Hb + Mb)] overshoot was calculated as the difference between the peak of the upward component and the asymptote of the downward component.

Arterialized capillary blood gas. A capillary blood sample (70 μL) was taken from participants' earlobe immediately after volitional exhaustion. Arterialization of capillary blood was achieved by applying a vasodilation cream (Capsolin; SIT s.r.l., Mede, Italy) before the test. Samples were immediately analyzed using an arterial blood gas analyzer (ABL-90 FLEX; Radiometer, Copenhagen, Denmark) for Hb concentration ([Hb]), pH, arterial partial pressure of CO_2 ($PaCO_2$), and $[La]_b$.

Calculated variables. Arterial partial pressure of O_2 (PaO_2) at peak was calculated from SpO_2 measurement at volitional exhaustion using the formula proposed by Severinghaus (35). Because both intense exercise and exposure to high altitude are known to alter $PaCO_2$ and acid–base homeostasis (36) thus alter the right or left shift of the O_2 dissociation curve, PaO_2 was corrected for both $PaCO_2$ and pH measured immediately after exercise cessation (35,37). Arterial O_2 content (CaO_2) at peak exercise was calculated using the direct measurement of [Hb] and SpO_2, an O_2 binding capacity of Hb of 1.34 mL $O_2 \cdot g \cdot Hb^{-1}$, a solubility of O_2 in the blood of 0.003 mL $O_2 \cdot dL\ blood^{-1} \cdot mm\ Hg^{-1}$, and the calculated PaO_2, as follows:

$$CaO_2 = [Hb] \times 1.34 \times \left(\frac{SpO_2}{100}\right) + 0.003 \times PaO_2 \quad [4]$$

A pioneering work (38) proposed a model in which $\dot{V}O_{2max}$ is not determined by one independent variable but rather the integration of both $\dot{Q}O_2$ and DO_2 components of O_2 transport. $\dot{Q}O_2$ was described by the Fick principle of mass conservation (39):

$$\dot{V}O_2 = \dot{Q} \times (a - vO_2diff) \quad [5]$$

where $\dot{Q}O_2$ and a-vO_2diff represent blood flow and arterial-venous O_2 difference, respectively. Likewise, DO_2 was calculated by rearranging the Fick law of diffusion:

$$\dot{V}O_2 = DO_2 \times (PcapO_2 - PmitoO_2) \quad [6]$$

where $PcapO_2$ and $PmitoO_2$ represent mean capillary and mitochondria partial pressure of oxygen (PO_2), respectively. A few important approximations are commonly considered to simplify the representation. First, this model described O_2 transport using a linear relationship between O_2 content and PO_2 in the blood (38). This might be true under hypoxic conditions where both arterial and venous values lie on the steep portion of the oxyhemoglobin dissociation curve. However, in normoxia, this assumption may cause a discrepancy from the results (38). Moreover, because at maximal exercise $PcapO_2$ has been demonstrated to be proportional to mixed venous PO_2 ($P\bar{v}O_2$) in both normoxia (40) and severe hypoxia (41), and $PmitoO_2$ is about 1–3 mm Hg (42) and is thus negligible, equation 6 can be simplified as follows (17,38):

$$\dot{V}O_2 = DO_2 \times k \times P\bar{v}O_2 \quad [7]$$

Similar to others (39), DO_2 was calculated as the slope of the $\dot{V}O_2$ over $P\bar{v}O_2$ relationship. Lastly, because a consistent number of investigations on both human and animal models demonstrated a high correlation between NIRS signals and mean venous O_2 saturation under different blood flow rates, fraction of inspired O_2, and exercise intensities (43,44), we assumed NIRS mTSI to be a relevant substitute to mean $PcapO_2$ for illustrative purposes (45).

Data Analyses

Mean values of $\dot{V}O_2$, HR, SVi, $\dot{Q}i$, and both muscle and cerebral NIRS data were calculated at relative work rates (40%, 60%, 80%, and 100% of the peak power output) to describe the changes in pulmonary, hemodynamic, and muscular and cerebral oxygenation patterns during incremental exercise. As previously described (46), Δ[deoxy(Hb + Mb)] kinetics during incremental exercise was assessed by fitting the Δ[deoxy(Hb + Mb)] versus workload relationship by a sigmoidal function of the type

$$y(x) = y_{bas} + A/\left[1 + e^{-c+dx}\right] \quad [8]$$

In equation 8, y_{bas} indicates the baseline, A the amplitude of the response, and c a constant dependent on d, which

represents the slope of the sigmoid, and where the ratio between c and d gives the x value corresponding to $(y_{bas} + A)/2$.

Statistical Analyses

All data are presented as mean ± SEM throughout the article. The required sample size was established *a priori* ($\alpha = 0.05$, $\beta = 0.80$) based on previous data from others (8) and by our research group (10) investigating the cardiorespiratory responses to exercise in hypoxia in preterm and term born adults.

A two-way (group–condition) mixed-effects ANOVA was performed to compare the cardiorespiratory, muscular, and cerebral oxygenation responses at rest and at each relative work-rate. Likewise, between-group and between-condition differences in arterialized capillary blood gas parameters and calculated variables were analyzed by a two-way (group–condition) mixed-effects ANOVA. Sidak *post hoc* corrections were examined in the event of interaction effects. Because HVR, lung function, and EIH were assessed only in normoxia, group differences in these variables were checked by a two-tailed Student's t test for unpaired data. Linear regression and correlation analyses were carried out by the least-squares residual method. All P values are two-tailed, and statistical significance was defined at the α level <0.05. Data analyses were performed using the statistical software package Prism v.6.0 (GraphPad Software, San Diego, CA).

RESULTS

None of the participants reported acute mountain sickness. Table 1 shows participants' characteristics, spirometry, and D_LCO parameters.

Adipose tissue thickness at the site of NIRS probe placement was similar between preterm and term born in both normoxia and hypoxia ($P = 0.992$ and $P = 0.831$, respectively).

Peak power output in normoxia was lower in preterm compared with term born (276 ± 10 vs 312 ± 12 W, $P = 0.042$) adults, whereas in hypoxia, both groups reached similar power output at maximal exercise intensity (248 ± 10 vs 279 ± 10 W, $P = 0.070$). However, the hypoxia-induced decrease in peak power output was similar between preterm and term born (Δ 30 ± 6 vs 33 ± 6 W, $P = 0.731$).

Acute transient hypoxic chemosensitivity. HVR was not different in preterm and term born adults (0.383 ± 0.061 vs 0.270 ± 0.029 $L \cdot min^{-1} \cdot \%^{-1}$; $r^2 = 0.80$ vs $r^2 = 0.75$, respectively; $P = 0.106$).

Gas exchange and EIH. At rest, hypoxia significantly increased V_T and $\dot{V}_E$, with a concomitant decrease in $P_{ET}CO_2$, in both preterm ($P = 0.002$, $P < 0.001$, and $P < 0.001$, respectively) and term born adults ($P = 0.013$, $P < 0.001$, and $P < 0.001$, respectively). GET was increased in hypoxia in both preterm and term born (68% ± 2% and 68% ± 1% $\dot{V}O_{2peak}$, respectively) participants compared with normoxia (62% ± 2% and 64% ± 2% $\dot{V}O_{2peak}$, respectively; condition effect: $P = 0.001$), whereas RCP was similar in both preterm and term born participants between normoxia (88% ± 2% and 88% ± 1% $\dot{V}O_{2peak}$, respectively) and hypoxia (87% ± 1% and 87% ± 1% $\dot{V}O_{2peak}$, respectively). Peak values for the main ventilatory and gas exchange variables are displayed in Table 2. Figure 1 shows $\dot{V}O_2$ kinetics at relative workloads. HVR was significantly correlated with relative $\Delta\dot{V}O_{2peak}$ in preterm ($r = 0.68$; $P = 0.005$) but not term born participants ($r = 0.077$; $P = 0.762$). EIH was evident in three preterm and three term born participants, without any difference in EIH severity between groups (87% ± 2% vs 88% ± 4%, respectively; $P = 0.798$). Breathing reserve was not different between groups and conditions. Wb and $VMRO_2$ were not different between preterm and term born adults (50 ± 16 vs 59 ± 17 $kg \cdot m^{-1} \cdot min^{-1}$ and 410 ± 120 vs 476 ± 126 $mL^{-1} \cdot min^{-1}$, respectively; $P = 0.084$ and $P = 0.084$), whereas both parameters were increased by hypoxia compared with normoxia (61 ± 16 vs 49 ± 16 $kg \cdot m^{-1} \cdot min^{-1}$ and 491 ± 118 vs 400 ± 119 $mL^{-1} \cdot min^{-1}$, respectively; $P < 0.001$ and $P < 0.001$).

TABLE 1. Physical characteristics, resting pulmonary function, and lung diffusion capacity in term born and preterm adults at sea level.

	Term Born	Preterm	P
Participants' characteristics			
Gestational age (wk)	40 ± 0	29 ± 1	<0.001
Birth weight (g)	3621 ± 101	1132 ± 64	<0.001
Age (yr)	21 ± 1	21 ± 1	0.066
Height (cm)	182 ± 2	178 ± 2	0.210
Body mass (kg)	75.6 ± 1.7	72.4 ± 3.5	0.415
BMI ($kg \cdot m^{-2}$)	22.8 ± 0.4	22.5 ± 0.7	0.713
Body surface area (m^2)	1.95 ± 0.03	1.89 ± 0.06	0.322
Pulmonary function			
FVC (L)	5.67 ± 0.17	5.37 ± 0.31	0.285
FVC (%predicted)	98 ± 2	98 ± 3	0.809
FEV_1 (L)	4.63 ± 0.18	4.23 ± 0.13	0.082
FEV_1 (%predicted)	93 ± 2	92 ± 2	0.412
FEV_1/FVC	0.82 ± 0.02	0.79 ± 0.01	0.261
FEV_1/FVC (%predicted)	92 ± 3	88 ± 5	0.167
PEF ($L \cdot s^{-1}$)	8.69 ± 0.37	8.83 ± 0.34	0.792
MVV ($L \cdot min^{-1}$)	195.0 ± 7.9	180.4 ± 6.9	0.172
MVV (%predicted)	106 ± 3	107 ± 3	0.820
DL_{CO} ($mL \cdot min^{-1} \cdot mm\ Hg^{-1}$)	35.1 ± 0.8[a]	31.8 ± 1.9[b]	0.121
DL_{CO} (%)	102 ± 3[a]	99 ± 5[b]	0.554
VA (L)	7.1 ± 0.2[a]	6.5 ± 0.3[b]	0.072
VA (%)	106 ± 2[a]	105 ± 3[b]	0.942
DL_{CO}/VA	5.0 ± 0.2[a]	4.9 ± 0.2[b]	0.671
DL_{CO}/VA (%)	97 ± 3[a]	93 ± 3[b]	0.453

[a]$n = 1$.
[b]$n = 2$.
BMI, body mass index; PEF, peak expiratory flow; VA, alveolar volume.

Cardiac and vascular hemodynamics. Systolic blood pressure was not different between groups and conditions, whereas diastolic blood pressure significantly increased in hypoxia in both preterm (from 81 ± 1 to 89 ± 2 mm Hg, $P = 0.004$) and term born (from 83 ± 2 to 92 ± 2 mm Hg, $P = 0.001$) participants. HR, SVi, and $\dot{Q}i$, kinetics from rest to peak exercise for both preterm and term born adults are shown in Figure 1, whereas peak values are reported in Table 2. The percent increase in SVi from 60% to 100% of peak power output was significantly different between preterm and term born in normoxia (−4.7% ± 3.0% vs +9.2% ± 2.7%, respectively; $P = 0.002$) but not in hypoxia (−6.0% ± 3.0% vs −3.4% ± 2.9%, respectively; $P = 0.541$). Similarly, in normoxia, $\dot{Q}i$ significantly increased from 80% to 100% of peak exercise intensity

TABLE 2. Cardiorespiratory, cardiac hemodynamics, and muscle and cerebral oxygenation responses at peak exercise intensity in normoxia and after 3-d high-altitude sojourn (3375 m).

	Normoxia		Hypoxia				
	Term Born	Preterm	Term Born	Preterm	Group	Condition	Group–Condition
$\dot{V}O_2$ ($L \cdot min^{-1}$)	3.93 ± 0.18	3.43 ± 0.16*	2.84 ± 0.09**	2.62 ± 0.11**	$P = 0.055$	$P < 0.001$	$P = 0.102$
$\dot{V}O_2$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	51.9 ± 1.9	48.5 ± 2.6	37.9 ± 1.1	36.4 ± 1.6	$P = 0.340$	$P < 0.001$	$P = 0.334$
$\dot{V}CO_2$ ($L \cdot min^{-1}$)	4.82 ± 0.20	4.16 ± 0.18	3.51 ± 0.12	3.27 ± 0.19	$P = 0.044$	$P < 0.001$	$P = 0.091$
$\dot{V}_E$ ($L \cdot min^{-1}$)	163.6 ± 6.9	145.5 ± 5.8	179.0 ± 5.8	169.8 ± 6.8	$P = 0.090$	$P < 0.001$	$P = 0.287$
Bf (breaths per minute)	59 ± 2	56 ± 3	61 ± 3	63 ± 2	$P = 0.632$	$P = 0.018$	$P = 0.314$
V_T (L)	2.803 ± 0.087	2.664 ± 0.090	2.968 ± 0.107	2.749 ± 0.111	$P = 0.212$	$P = 0.005$	$P = 0.569$
$P_{ET}CO_2$ (mm Hg)	32 ± 1	32 ± 1	24 ± 0	23 ± 0	$P = 0.517$	$P < 0.001$	$P = 0.853$
$[La]_b$ ($mmol \cdot L^{-1}$)	14.6 ± 0.8	14.3 ± 0.9	15.8 ± 0.8	13.5 ± 1.0	$P = 0.137$	$P = 0.878$	$P = 0.192$
RPE	19 ± 0	19 ± 0	19 ± 0	18 ± 0	$P = 0.111$	$P = 0.189$	$P = 0.588$
SpO_2 (%)	96 ± 1	94 ± 1	83 ± 1	85 ± 1	$P = 0.915$	$P < 0.001$	$P = 0.068$
HR (bpm)	189 ± 3	188 ± 2	190 ± 2	186 ± 2	$P = 0.556$	$P = 0.750$	$P = 0.190$
HR (%predicted HR)	98 ± 1	97 ± 1	98 ± 1	96 ± 1	$P = 0.476$	$P = 0.759$	$P = 0.138$
SVi ($mL \cdot m^{-2}$)	71 ± 3	61 ± 3*	61 ± 2**	61 ± 3	$P = 0.087$	$P = 0.041$	$P = 0.045$
$\dot{Q}i$ ($L \cdot min^{-1} \cdot m^{-2}$)	13.3 ± 0.5	11.4 ± 0.5*	11.6 ± 0.4**	11.2 ± 0.5	$P = 0.029$	$P = 0.031$	$P = 0.054$
mTSI (%)	56.0 ± 1.4	54.4 ± 2.3	53.5 ± 1.7	54.9 ± 2.0	$P = 0.855$	$P = 0.467$	$P = 0.207$
Δ[oxy(Hb + Mb)] (μM)	−11.98 ± 1.19	−14.81 ± 1.44	−22.66 ± 1.17	−23.48 ± 1.83	$P = 0.197$	$P < 0.001$	$P = 0.369$
Δ[deoxy(Hb + Mb)] (μM)	11.04 ± 1.53	13.46 ± 2.48	16.17 ± 2.06**	13.65 ± 1.98	$P = 0.837$	$P = 0.120$	$P = 0.046$
Δ[oxy(Hb + Mb) + deoxy(Hb + Mb)] (μM)	−0.39 ± 2.26	−0.49 ± 3.00	−6.49 ± 1.85	−9.83 ± 2.39	$P = 0.534$	$P < 0.001$	$P = 0.393$
bTSI (%)	67.8 ± 1.2	68.6 ± 1.4	63.1 ± 1.2	66.7 ± 1.3	$P = 0.164$	$P = 0.001$	$P = 0.125$
Δ[oxyHb] (μM)	10.39 ± 1.87	9.78 ± 1.92	1.10 ± 1.90	0.48 ± 2.11	$P = 0.849$	$P < 0.001$	$P = 0.918$
Δ[deoxyHb] (μM)	5.78 ± 0.74	5.63 ± 0.85	10.91 ± 1.20	9.89 ± 1.11	$P = 0.596$	$P < 0.001$	$P = 0.577$
Δ[oxyHb+deoxyHb] (μM)	16.17 ± 1.71	15.42 ± 2.20	12.01 ± 2.63	10.37 ± 2.76	$P = 0.711$	$P = 0.013$	$P = 0.855$

*$P < 0.05$ different from term born.
**$P < 0.05$ different from normoxia.
Bf, breathing frequency; bTSI, brain tissue saturation index; $[La]^-$, blood lactate concentration; RPE, rate of perceived exertion; $\dot{V}CO_{2peak}$, carbon dioxide production.

in term born compared with preterm (+15.0% ± 2.5% vs +3.6% ± 2.4%, respectively; $P = 0.009$) adults, whereas no difference between groups was found in hypoxia (−0.4% ± 1.5% vs +2.9% ± 2.5%, respectively; $P = 0.618$).

Muscular and cerebral oxygenation. During the "physiological" calibration, both groups reached similar levels of deoxygenation (Δ[deoxy(Hb + Mb)]) in both normoxia and hypoxia (preterm: 17.9 ± 1.3 and 17.3 ± 1.1 μM, respectively; term born: 17.9 ± 1.7 and 17.5 ± 1.3 μM, respectively; group effect: $P = 0.956$, condition effect: $P = 0.523$, group–condition effect: $P = 0.985$). Kinetics of muscle and brain NIRS parameters from rest to peak exercise for both groups are shown in Figures 2 and 3, respectively. Peak values are reported in Table 2. In normoxia, a significantly higher Δ[deoxy (Hb + Mb)] from rest to 40% of peak power output was observed in preterm compared with term born (8.74 ± 1.86 vs 4.17 ± 0.60 μM, respectively; $P = 0.020$) participants.

Intramuscular matching between O_2 delivery and O_2 utilization. Figure 4A depicts the Δ[deoxy(Hb + Mb)] overshoot of a typical participant (*term born*, *hypoxia*) during the first 2 min of very low intensity exercise (60 W). In normoxia, the majority of participants (11 of 17 in each group) showed an overshoot in Δ[deoxy(Hb + Mb)], whereas in hypoxia, this was evident only in four preterm and two term born adults. Group means and SEM of the Δ[deoxy(Hb + Mb)] overshoot are presented in Figure 4B.

Arterialized capillary blood gas and calculated variables. $[La]_b$ values at peak exercise are shown in Table 2. At peak, [Hb] and pH were similar between groups and conditions. As expected, PaO_2 was significantly lower in hypoxia compared with normoxia in both preterm (48 ± 2 vs 75 ± 9 mm Hg, respectively; $P = 0.006$) and term born (44 ± 2 vs 88 ± 6 mm Hg, respectively; $P < 0.001$) participants, without any difference between groups ($P = 0.842$ and $P = 0.222$, respectively). Similarly, $PaCO_2$ was significantly lower in hypoxia compared with normoxia in both preterm (25.2 ± 0.9 vs 34.1 ± 1.5 mm Hg, respectively; $P < 0.001$) and term born (24.5 ± 0.8 vs 33.3 ± 1.8 mm Hg, respectively; $P < 0.001$) participants, without any difference between groups ($P = 0.921$ and $P = 0.906$, respectively). Hypoxia decreased CaO_2 in term born (Δ −3.9 ± 0.7 $mL \cdot dL^{-1}$, $P < 0.001$) but not in preterm (Δ −2.1 ± 1.1 $mL \cdot dL^{-1}$, $P = 0.116$) adults, without any difference between groups in both normoxia (24.8 ± 0.5 vs 24.6 ± 0.8 $mL \cdot dL^{-1}$, respectively; $P = 0.959$) and hypoxia (20.9 ± 0.6 vs 22.7 ± 0.6 $mL \cdot dL^{-1}$, respectively; $P = 0.159$). $\dot{Q}O_2$ and DO_2 changes from normoxia to hypoxia in preterm and term born are shown in Figures 5A and B, respectively. Interestingly, a lower $\dot{Q}O_2$ was observed in preterm compared with term born (5323 ± 389 vs 6389 ± 271 $mL \cdot min^{-1}$, $P = 0.040$) in normoxia, but not in hypoxia (4996 ± 412 vs 4732 ± 226 $mL \cdot min^{-1}$, $P = 0.960$; Fig. 5A). In normoxia, a positive correlation was observed between $\dot{Q}O_2$ and relative $\dot{V}O_{2peak}$ in term born ($r = 0.55$, $P = 0.033$), but not in preterm, adults.

DISCUSSION

This study investigated the cardiorespiratory, hemodynamics, and muscular and cerebral oxygenation responses to maximal exercise performed in normoxia and at high-altitude (hypobaric hypoxia) in prematurely and term born healthy adults. The general aim was to identify the mechanisms and sites of exercise capacity limitation in prematurely born but otherwise healthy individuals and to clarify whether altitude exposure may exacerbate potential hypoxia-related impairment(s). To the best of our knowledge, this article is the first to simultaneously investigate the cardiac hemodynamic, muscle, and cerebral oxygenation kinetics from rest to peak exercise intensity in preterm adults under both normoxic and

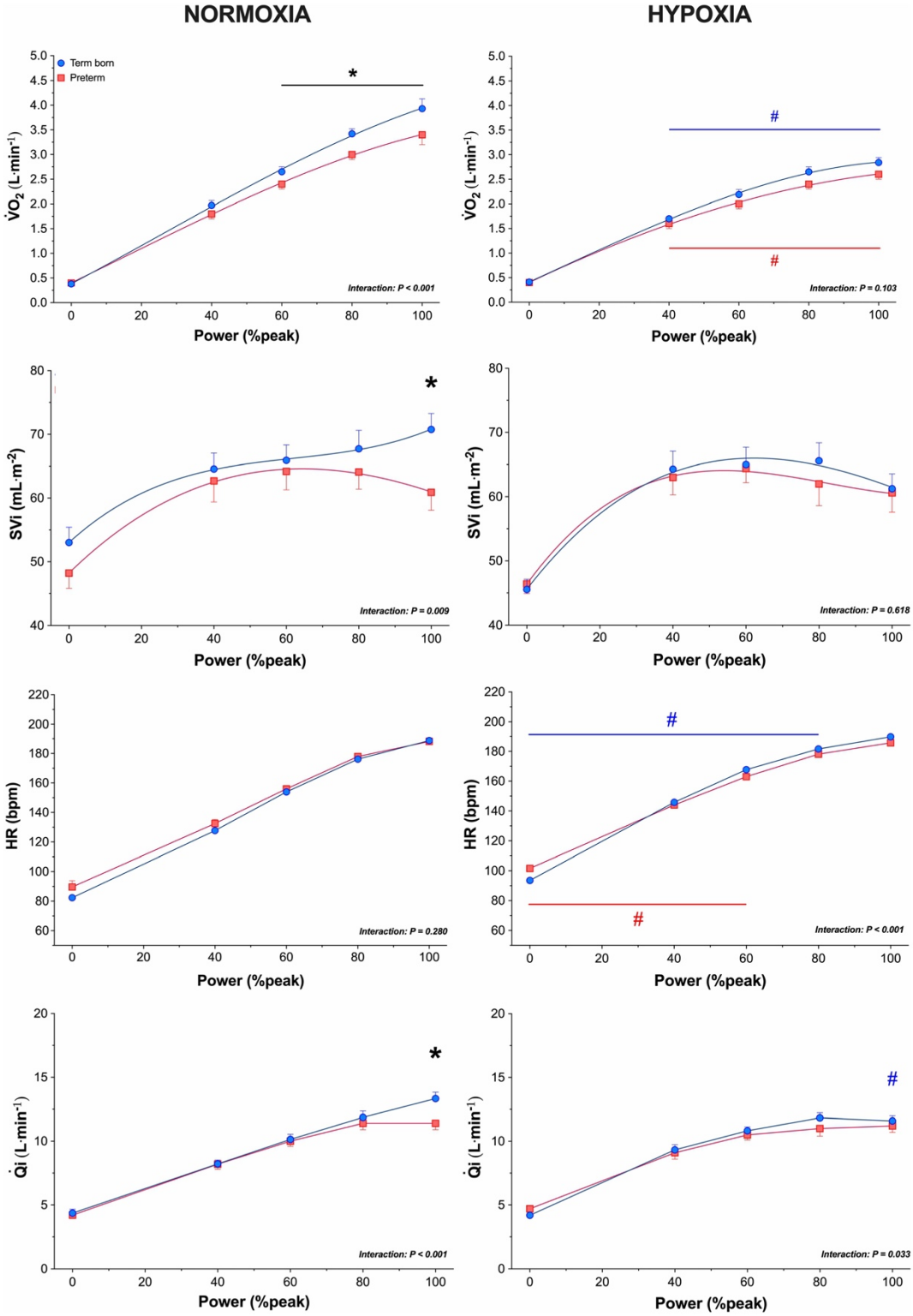

FIGURE 1—Pulmonary O_2 uptake and cardiac hemodynamics changes from rest to peak exercise intensity during normoxic and hypoxic progressive exercise test (plotted as increasing percent of maximal power output reached at volitional exhaustion) in prematurely born (*red squares*) and term born (*blue circles*) adults. Values are shown as mean ± SEM. *Significantly different from term born. #Significantly different from the same normoxic relative workload.

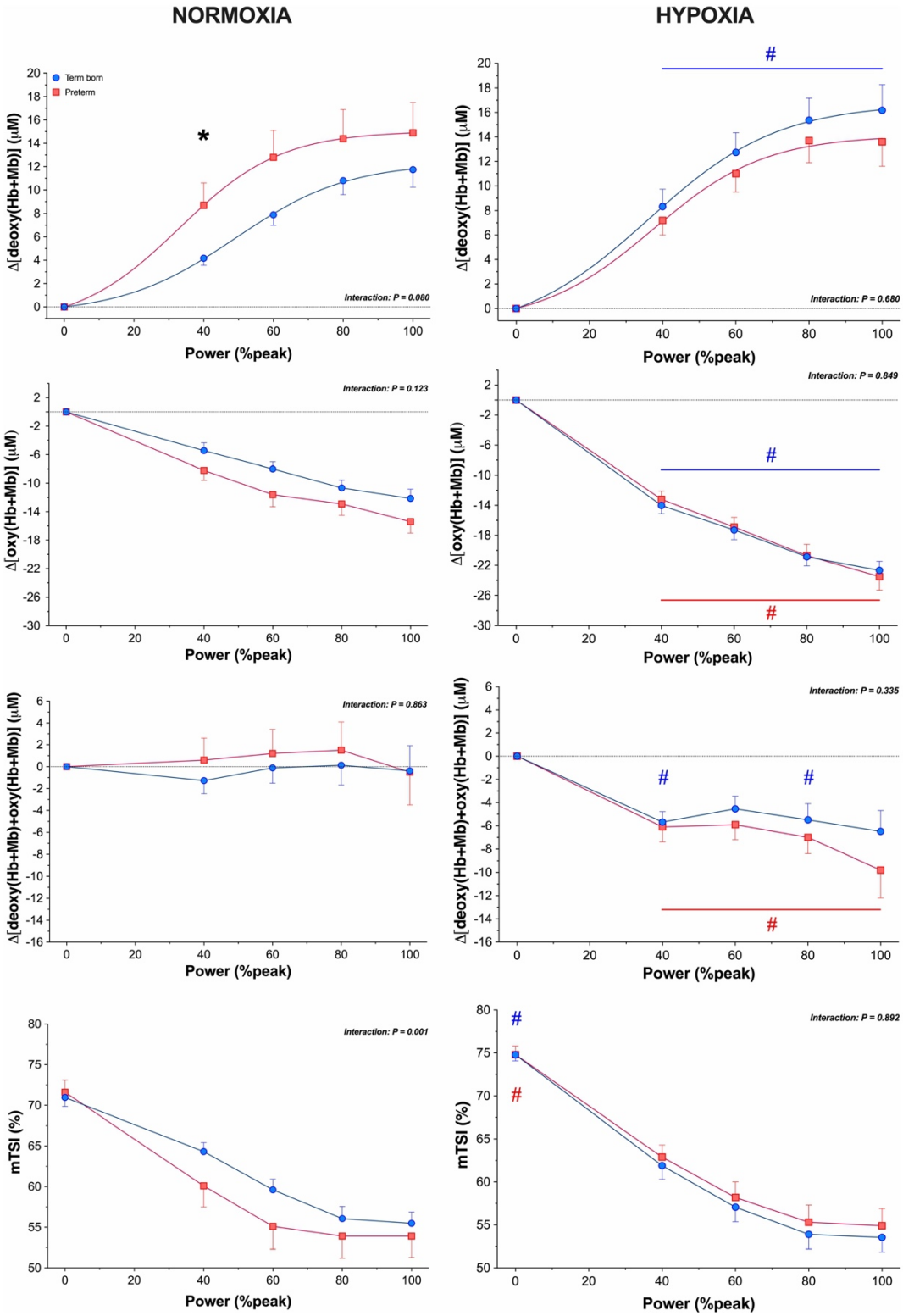

FIGURE 2—Muscle NIRS-derived parameters changes during normoxic and hypoxic progressive exercise test to volitional exhaustion (plotted against percent of peak power output) in prematurely born (*red squares*) and term born (*blue circles*) adults. Data are mean ± SEM. *Significantly different from term born. #Significantly different from the same normoxic relative workload.

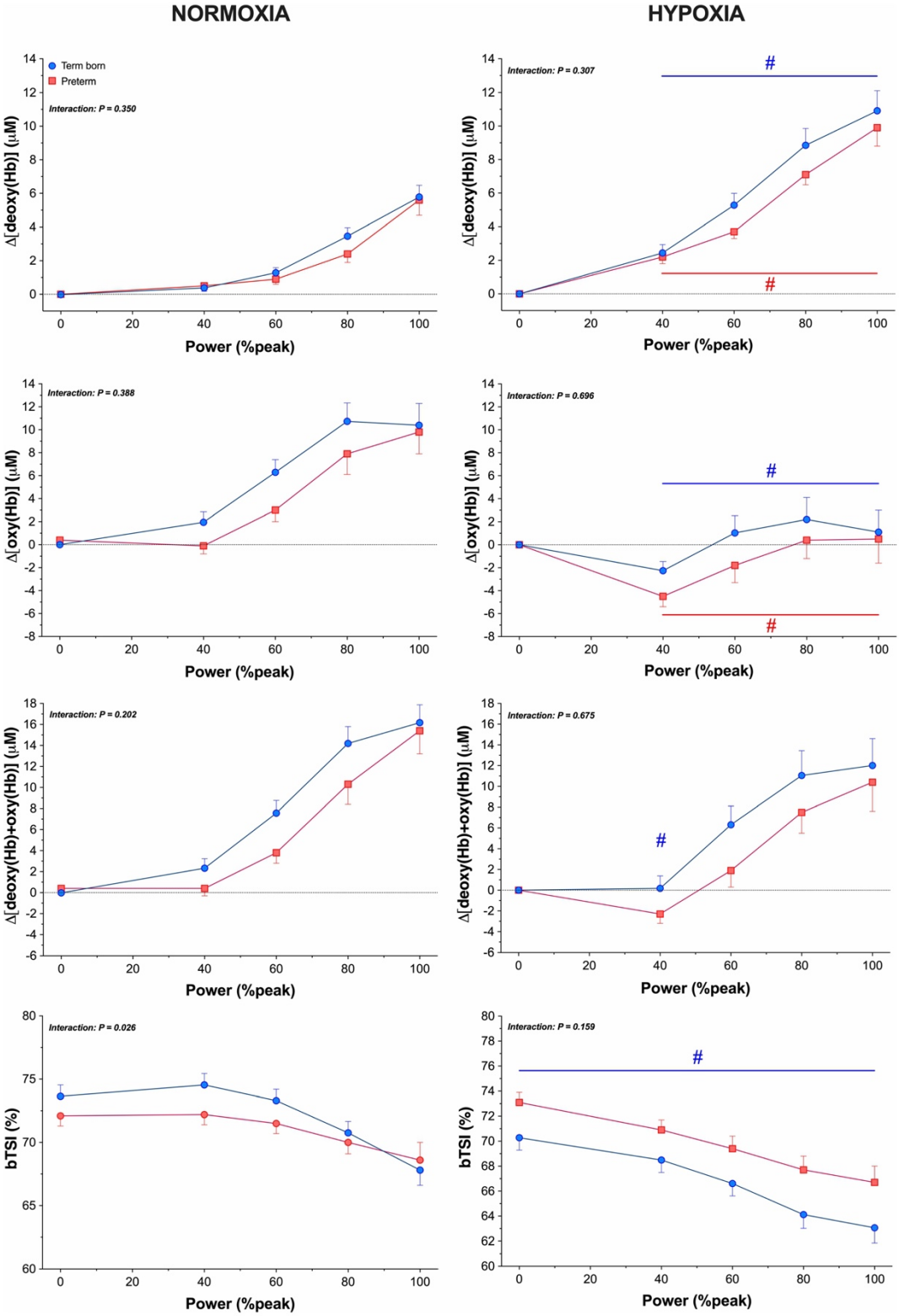

FIGURE 3—Cerebral oxygenation and deoxygenation changes recorded by NIRS during normoxic and hypoxic incremental exercise test to volitional exhaustion (plotted against percent of peak power output) in prematurely born (*red squares*) and term born (*blue circles*) adults. Data are mean ± SEM. #Significantly different from the same normoxic relative workload.

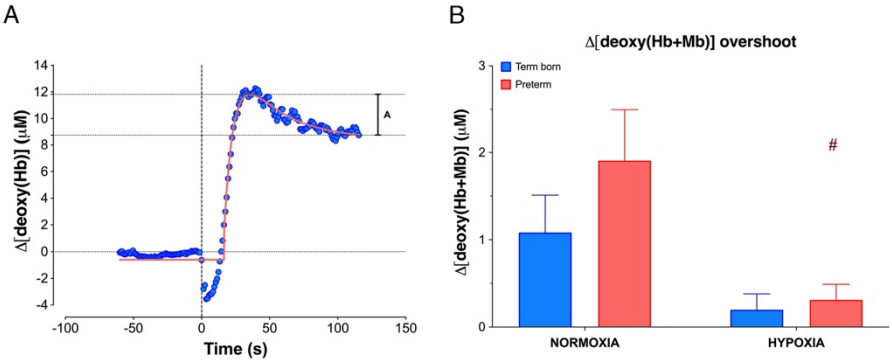

FIGURE 4—Intramuscular matching between O_2 delivery and O_2 utilization in the transition from rest to low-intensity exercise. Panel A shows a typical example of [deoxy(Hb + Mb)] changes (*blue circles*) from the last 60 s of rest to the first 120 s of low-intensity constant work rate exercise (60 W). The presence of a transient [deoxy(Hb + Mb)] overshoot, an indirect estimation of intramuscular matching between O_2 delivery and O_2 utilization, was checked by fitting the NIRS data by a double exponential function (*solid red line*). The amplitude (*A*) of this deoxygenation overshoot was calculated as the difference between the asymptotes of the two equations (*horizontal dotted lines*). Panel B shows the group mean (±SEM) of the NIRS deoxygenation overshoot in both normoxia and hypobaric hypoxia in preterm (in *red*) and term born (in *blue*) adults. #Significantly different from the same normoxic relative workload.

hypoxic conditions. The obtained data indicate that in prematurely born adults with normal lung function breathing normoxic air, the main limitation is located in the cardiovascular system, as highlighted by the different SVi kinetics from 60% of peak power to the point of volitional exhaustion. In contrast with our initial hypothesis, however, muscle oxygenation was not different between preterm and term born adults during exercise. This highlights that cardiovascular rather than diffusive mechanisms at muscle level underlie the reduced exercise capacity reported in the present study as well as by a growing number of other investigations (7–10). Moreover, despite preliminary evidence on animal models (16) and preterm kids (47), the present work is the first to demonstrate a lower sensitivity to the detrimental effects of hypoxia in preterm adults on exercise cardiac hemodynamics compared with their term born peers.

The majority of the literature agrees that premature birth *per se*, or in conjunction with pulmonary diseases (i.e., bronchopulmonary dysplasia), is associated with several physiological alterations which ultimately limit exercise capacity in normoxia (1,6–10). This is also confirmed in the present study where, despite similar $\dot{V}O_{2peak}$, peak power output was 11% lower in preterm compared with term born. Although the exact underlying mechanisms are still not completely clear, current consensus suggests that cardiopulmonary dysanapsis might chiefly underpin exercise intolerance in prematurity (1,5). The preterm group investigated in the present study had normal resting lung function and diffusion capacity, as well as similar $\dot{V}_E$ at the point of volitional exhaustion compared with term born. Data on impaired pulmonary function at rest are contradictory, with reduced (9) or unchanged (3,8,10) lung function in preterm compared with their term born counterparts. Similarly, D_LCO has been shown to be either reduced (9) or unchanged in preterm cohorts (8). Our data suggest that healthy and moderately active prematurely born adult may not present largely impaired resting pulmonary function and diffusion capacity. Recent work also reported minor lung function abnormalities in 11-yr-old children born preterm in the turn of the millennium compared with those born in the early 1990s (48), suggesting a reexamination of the currently accepted paradigm of deleterious long-term respiratory consequences induced by premature birth could be necessary. Intriguingly, lung function indices were similar between 11-yr-old term born controls and age-matched preterm born children in the late 1990s (48).

Recent imaging works focused on structural and functional cardiac changes from premature birth to young adulthood indicate cardiac remodeling in both the right and left ventricles (4,49). Overall, the preterm heart presents smaller ventricular internal dimensions and impaired systolic, diastolic, and rotational functions (4,49), and these alterations seem to be exacerbated by physiological stressors such as exercise (50). Our data indicating lower SVi at peak exercise leading to decreased $\dot{Q}i$ at volitional exhaustion strongly support these previous findings and highlight the intrinsic function of the heart as the potential limiting factor to exercise capacity in preterm adults without significant pulmonary function impairments. Although SVi was significantly lower at exhaustion, no difference was observed at submaximal intensities. Despite this, from 60% to 100% of peak exercise intensity, preterm showed a downward kinetics in SVi compared with the upward trend found in term born participants. This underlines that exercise capacity in preterm strays from term born when exercise intensity increases slightly above GET. A recent study performed stress echocardiography during submaximal exercise bouts at the same relative workloads used in the present study to investigate left ventricular function (50). In line with our results, left ventricular function was impaired in preterm adults at rest, and ejection fraction was significantly lower at both 60% and 100% of peak exercise intensity in preterm born compared with term born participants (50). These findings support previous animal models of preterm birth, which showed prematurity-induced cardiac alterations and systolic deficits (51) that, according to our results, become evident even at submaximal exercise in a cohort of

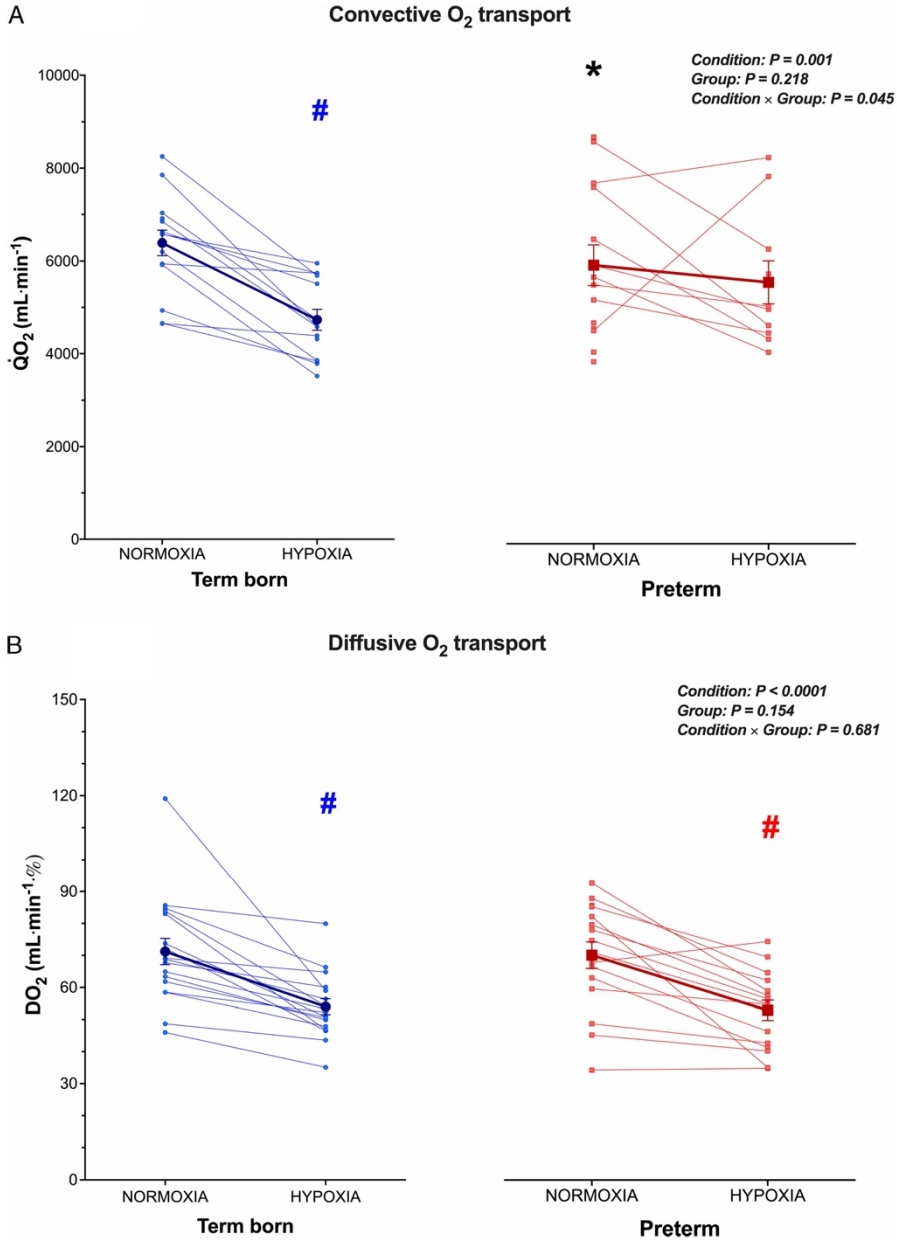

FIGURE 5—Group mean (±SEM) and individual values changes in convective (A) and diffusive (B) components of O_2 transport from normoxia to hypobaric hypoxia in prematurely born (*red squares*) and term born (*blue circles*) adults. *Significantly different from term born. #Significantly different from the same normoxic relative workload.

young, moderately active and normotensive prematurely born adults.

Literature on the contribution of skeletal muscle and cerebral oxygenation on exercise capacity in prematurely born adults is still scarce. An increased O_2 extraction was observed in preterm children during submaximal exercise (6-min walking test) compared with their term born counterparts (52). Therefore, it might be argued that exercise capacity in the moderate-intensity domain is not reduced in preterm compared with term born adults because of an increased O_2 utilization at the skeletal muscle level. However, the intramuscular matching between O_2 delivery and O_2 uptake, assessed by analyzing the time course of Δ[deoxy(Hb + Mb)] during the first 2 min of low-intensity constant work rate cycling exercise (34), was similar between groups implying that matching between intramuscular O_2 delivery and metabolic demand is not impaired by prematurity. Cerebral oxygenation and deoxygenation in both normoxia and hypoxia were similar between preterm and term born participants at every relative work rate. According to a previous investigation

(53), our results indicate that cerebral oxygenation did not limit exercise capacity under both normoxic and hypoxic conditions in our groups. Interestingly, hypoxia decreased cerebral oxygenation both at rest and during exercise in term born but not in preterm, adults, supporting earlier speculations (53) of a more pivotal role of cerebral oxygenation to limit exercise performance in hypoxia in term born participants. However, it is important to highlight that the hypoxic level in this latter study (P_IO_2 ~ 75 mm Hg) was slightly higher than in the present one (P_IO_2 ~95 mm Hg).

In the present study, chemosensitivity to hypoxia was evaluated using transient full nitrogen breathing periods (20). Analyses of $\dot{V}_E$ over SpO_2 revealed that preterm adults have similar HVR to their counterparts born at term. Unexpectedly, these results contrast with previous reports (10,14) where HVR was significantly reduced in preterm compared with term born adults probably because of peripheral chemoreceptor disruption induced by perinatal O_2 therapy (14). However, the differences between our results and those of the latter studies (10,14) could be explained by the biphasic ventilatory response to hypoxia described in prematurely born infants (54). Hypoxia triggered an initial exaggerated stimulation of peripheral chemoreceptors in preterm infants, which, after 3 min, was overridden by a decline in $\dot{V}_E$ secondary to hypoxemia-induced depression of central respiratory centers activity (54). Hence, although the protocol used in the present study exposed the preterm participants to transient (<1 min) hypoxic periods, others (10,14) assessed HVR during longer (≥4 min) hypoxic stages. Although caution is needed when comparing our findings with previous ones (10,14) because of the different durations of hypoxic exposure, our results suggest that this unique resting biphasic ventilatory response to hypoxia persists into adulthood.

Recent evidence from human and animal studies of premature birth with postnatal O_2 therapy and hyperoxic treatment, respectively, demonstrated preserved cardiac function and exercise capacity in hypoxia during adulthood compared with their peers born at term (8,16). In contrast, however, a recent cardiac imaging study reported an exaggerated resting cardiac contractile response, especially at the level of the right ventricle, in adults born preterm, compared with their term born counterparts, when exposed to acute normobaric hypoxia (55). In the present study, in normoxia, both peak power output and absolute $\dot{V}O_{2peak}$ were significantly lower in preterm compared with term born, whereas in hypoxia, they reached similar values at maximal exercise intensity, hence increasing evidence on lower hypoxia-induced impairments during exercise in adult survivors of premature birth. These later results contradict our initial hypothesis of larger effects of hypoxia on any deficiency in exercise capacity that preterm adults often show during strenuous exercise under normoxic conditions. Unexpectedly, the differences between preterm and term born adults observed in normoxia were reduced under hypoxic conditions because preterm had significantly lower $\dot{V}O_{2peak}$, SVi, and $\dot{Q}i$ at exhaustion in normoxia, but not in hypoxia. Also, adults born preterm exhibited a significantly lower Δ[deoxy(Hb + Mb)] overshoot in hypoxia compared with normoxia indicating a better match between metabolic demand and O_2 delivery.

Figure 6 attempts to explain these concepts by depicting the relative contribution of $\dot{Q}O_2$ (sigmoid line) and DO_2 (straight

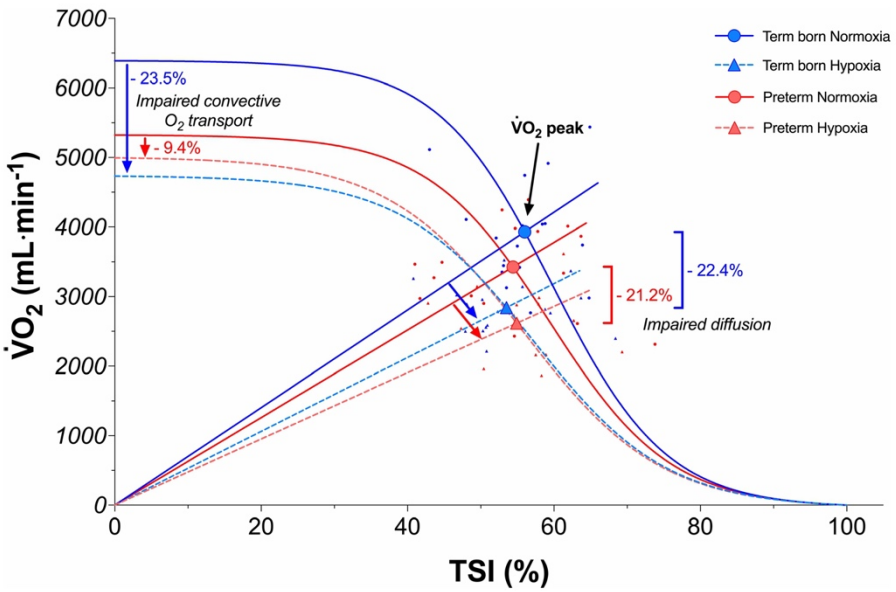

FIGURE 6—Graphical representation of the relative contribution of convective (*sigmoid lines*) and diffusive (*straight lines* from the origin) components of O_2 transport in prematurely born (in *red*) and term born (in *blue*) adults at sea-level (*solid lines*) and high-altitude (3375 m, *dotted lines*) that integrate to determine $\dot{V}O_{2max}$. Convection can be mathematically described by the Fick principle of mass conservation, whereas O_2 diffusion by the Fick law of diffusion. The intersection between these two relationships represents $\dot{V}O_{2max}$. See Methods for further details. Individual normoxic and hypoxic $\dot{V}O_{2max}$ values are plotted for each group. TSI refers to muscle tissue saturation index.

line) as central and peripheral components of O_2 transport (see Methods), respectively; in preterm and term born adults under both normoxic and hypoxic conditions (17,38). It is important to acknowledge that, based on previous findings (43–45), we assumed mTSI to be analogous to mean $PcapO_2$ for illustrative purposes (see Methods). This analysis suggests that in normoxia, a large difference in $\dot{Q}O_2$ exists between preterm and term born adults, whereas DO_2 remains similar. However, because the DO_2 component includes oxygen diffusion at both pulmonary and muscle levels (17), future investigations should investigate the relative contribution of both compartments in this population. Moreover, the larger degree of hypoxia-induced perturbation in cardiorespiratory, muscle, and cerebral oxygenation responses to exercise observed in the present cohort of term born adults can be explained by a twofold decrease in $\dot{Q}O_2$ compared with preterm participants (−23.5% vs −9.4%), whereas DO_2 decreased in both groups in a similar way.

Methodological considerations. In an expedition setting at extreme altitude, despite efforts to carefully standardize environmental factors, some risk of bias remains. First, the preterm cohort investigated in the present study had normal lung function and relatively high $\dot{V}O_{2peak}$ compared with the values usually reported in the literature for this population (9). However, this may be a direct consequence of our aim to compare two groups of recreationally active participants to have similar degrees of hypoxia-induced decreases in $\dot{V}O_{2peak}$. Likewise, pulmonary function in the preterm participants was within the normal range, and according to recent findings (48), this might be a consequence of them being born at the turn of the millennium with better neonatal management, which improves long-term pulmonary outcomes. Second, because the present study investigated only male participants, these results cannot be directly extended to female participants because menstrual cycle and the associated hormonal changes are known to influence the physiological responses to hypoxia both at rest and during exercise (56). However, because preliminary results suggest sex differences in the physiological responses to both rest and exercise in preterm adults (4,57), we want to highlight the need for further research in both sexes. In addition, PaO_2 values were corrected for measured pH and $PaCO_2$ at the point of volitional exhaustion, but not for body temperature because this parameter was not measured in the present study. Finally, the calculation of DO_2 was based on whole-body measurements of $\dot{V}O_2$ and $\dot{Q}$, and it is well known that muscle blood flow is slightly lower than $\dot{Q}$; hence, our calculation of DO_2 may be overestimated (39). Also, similarly to previous authors (45), we used mTSI as an indirect determination of mean $PcapO_2$. Although experimental data suggest NIRS parameters to be associated with mixed venous O_2 content (43,44) and that this latter is linearly proportional to mean $PcapO_2$ (40), this assumption may have introduced variability given that NIRS measurements might not record changes at capillary or venous compartments only. However, the use of NIRS TSI to understand the integration of diffusive and convective components of O_2 transport is currently under debate, and it might represent a useful alternative to gold standard but more invasive techniques that directly measure arterial and venous PO_2.

CONCLUSIONS

Overall, the present results suggest that long-term effects of premature birth impair exercise capacity in normoxia by primarily limiting convective mechanisms in O_2 transport. Future work should investigate the specific site of cardiac limitation and potential pharmacological and nonpharmacological interventions, which may overcome these impairments and ultimately increase exercise capacity in this population under normoxic conditions. However, preterm adults exposed to hypobaric hypoxia exhibited lower sensitivity to the detrimental effects induced by lower inspired O_2 concentration on cardiorespiratory, muscle, and cerebral oxygenation responses to incremental exercise tests to exhaustion. In conclusion, premature birth and/or perinatal cares might contribute to a "hypoxic preconditioning" effect, at least partly in male participants and during adulthood. Investigation of the effects of the modern O_2 therapy of preterm newborns is also warranted.

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10. Article 2

Microvascular and oxidative stress responses to acute high-altitude exposure in prematurely born adults

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Microvascular and oxidative stress responses to acute high-altitude exposure in prematurely born adults

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Premature birth is associated with endothelial and mitochondrial dysfunction, and chronic oxidative stress, which might impair the physiological responses to acute altitude exposure. We assessed peripheral and oxidative stress responses to acute high-altitude exposure in preterm adults compared to term born controls. Post-occlusive skeletal muscle microvascular reactivity and oxidative capacity from the muscle oxygen consumption recovery rate constant (*k*) were determined by Near-Infrared Spectroscopy in the *vastus lateralis* of seventeen preterm and seventeen term born adults. Measurements were performed at sea-level and within 1 h of arrival at high-altitude (3375 m). Plasma markers of pro/antioxidant balance were assessed in both conditions. Upon acute altitude exposure, compared to sea-level, preterm participants exhibited a lower reperfusion rate (7 ± 31% vs. 30 ± 30%, $p = 0.046$) at microvascular level, but higher *k* (6 ± 32% vs. −15 ± 21%, $p = 0.039$), than their term born peers. The altitude-induced increases in plasma advanced oxidation protein products and catalase were higher (35 ± 61% vs. −13 ± 48% and 67 ± 64% vs. 15 ± 61%, $p = 0.034$ and $p = 0.010$, respectively) and in xanthine oxidase were lower (29 ± 82% vs. 159 ± 162%, $p = 0.030$) in preterm compared to term born adults. In conclusion, the blunted microvascular responsiveness, larger increases in oxidative stress and skeletal muscle oxidative capacity may compromise altitude acclimatization in healthy adults born preterm.

Preterm birth affects over 10% of live births worldwide, and those born moderately to extremely preterm (≤ 32 weeks gestation) are considered at highest risk for short- and long-term respiratory and cardiovascular morbidity[1]. Though a growing number of preterm birth survivors reach adulthood, increasing evidence supports several long-term sequelae of early delivery on the cardiovascular system[2,3], reducing exercise capacity[4] and ultimately leading to increased cardiovascular and metabolic disease risk[5]. Given that several of these comorbidities have been associated with mitochondrial dysfunction and chronic oxidative stress[6], it is reasonable to hypothesize that premature birth evokes specific peripheral responses to certain stimuli[7].

Previous attempts to investigate vascular health in young adults born preterm have provided equivocal results. For example, endothelial function assessed by flow-mediated dilation or finger plethysmography was reported as normal[5,8] or reduced[2,9]. In contrast, both microvascular function and density were found to be reduced in adults born prematurely[3].

Premature birth also seems to impact redox balance, likely as a consequence of perinatal supplemental oxygen therapy[10]. However, the results of these investigations are also not consistent. Systemic oxidative stress markers have been reported to be higher (e.g., 8-isoprostane in exhaled breath condensate, and total superoxide dismutase (SOD) and glutathione peroxidase (GPX) in blood)[10] or similar (e.g., advanced oxidation protein

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products (AOPP), Catalase and SOD in plasma)[11] in preterm adults compared to term born peers. Given that oxidative stress is known to be modulated by environmental hypoxia[11] and, simultaneously, plays a central role in the individual response to hypoxia[10], a specific phenotypical response to hypoxia may be present in prematurely born adults. However, to date, findings regarding the systemic and localized effects of acute hypobaric hypoxia on redox balance modulation and microvascular reactivity in this population remain scarce.

Oxidative stress is also known to be a potent modulator of oxidative capacity and metabolic responses at the skeletal muscle level in adults born preterm[10]. Animal models used to simulate premature birth by postnatal hyperoxic exposure have demonstrated alterations in muscle and mitochondrial functions[12]. These finding where recently extended to adult humans born prematurely, where mitochondrial function in peripheral blood mononuclear cell was increased compared to control peers born at term[13]. However, despite these preliminary findings, no in vivo evidence on skeletal muscle oxidative function is currently available in preterm individuals.

Near-infrared spectroscopy (NIRS) in association with vascular occlusion testing (VOT) has recently emerged as a non-invasive method of evaluating resting muscle oxygen consumption and microvascular reactivity[14,15], as well as muscle oxidative capacity[16–18]. A blunted microvascular hyperemic response following an ischemic stimulus has been previously reported in diseased populations with cardiovascular impairments[19] and in sedentary (relative to endurance-trained) individuals[20,21]. Similarly, skeletal muscle oxidative capacity was found to be significantly lower in patients with pulmonary[22] and cardiovascular diseases[23]; conditions associated with reduced exercise capacity. A recent mechanistic study assessed skeletal muscle O_2 diffusion capacity (DmO_2) by evaluating skeletal muscle oxidative capacity under high and low levels of tissue O_2 availability in the *vastus lateralis* muscle[18]. This technique was subsequently proposed to also be applicable with the use of systemic hypoxia; a decreased whole-body O_2 supply was proposed as a surrogate for the low oxygen availability conditions that allow DmO_2 computation[24].

Accordingly, the aim of this study was to investigate the acute peripheral (microvascular reactivity and skeletal muscle oxidative capacity) and systemic oxidative stress responses to high-altitude exposure in healthy prematurely born adults and their age-matched term born peers. We tested the hypotheses that (i) preterm adults would demonstrate impaired microvascular reactivity and skeletal muscle oxidative capacity, and that (ii) acute high-altitude exposure would reduce these impairments relative to their term born peers.

Methods

Participants. Thirty-four young healthy men volunteered and gave written informed consent to participate in this study. Seventeen participants were born preterm. Participants were matched for age and fitness status. All participants were not taking any medication and were free from any cardiorespiratory, neurological and hematological diseases. Moreover, none of the participants was a habitual smoker. The preterm born participants were recruited via the National preterm birth register managed by the University Clinical Centre in Ljubljana, Slovenia using medical record screening and telephone/email-based individual interviews. Importantly, the included preterm participants had a gestational age ≤ 32 weeks, a birth weight ≤ 1500 g, had received postnatal supplemental oxygen therapy, and none of the participants had a history of bronchopulmonary dysplasia. The inclusion criteria for the term born participants were gestational age ≥ 38 weeks and birth weight ≥ 2500 g. Birth-related inclusion criteria for all participants were checked and confirmed during the initial birth/medical record screening procedure conducted prior to inclusion in the study. The experimental protocol was pre-registered at ClinicalTrials.gov (NCT04739904), approved by both the University of Ljubljana, Faculty of Sport ethics committee (8/2020-316) and the Aosta Hospital Ethical Committee (06/05/2021.0038781.I), and performed in line with the Declaration of Helsinki guidelines.

Experimental design and ascent protocol. Each participant underwent two experimental trials, the first one near sea-level (Ljubljana, Slovenia; barometric pressure ~ 737 ± 0.5 mmHg) and the other one within 1 h of arrival at high-altitude (3375 m; Torino hut, Aosta Valley, Italy, on the Mont Blanc massif; barometric pressure ~ 503 ± 0.7 mmHg). Subjects traveled to Courmayeur (1300 m) by car, then traveled by cable car to Torino hut in 15–20 min. On average, the duration between the two phases was 97 ± 4 days. By performing the sea-level measurements prior to the high-altitude exposure, we ensured that there were no potential carryover effects of altitude testing on the subsequent control tests, as would have been the case for some participants in a randomized crossover design. During the informed consent visit, participant performed a cycling exercise test and a 5-min leg occlusion to familiarize with the testing procedures. Blood pressure was measured on the left arm at rest both at sea-level and upon arrival to altitude using a digital sphygmomanometer (M2, OMRON Healthcare, Hoofddorp, The Netherlands).

NIRS-VOT-derived muscle oxygenation and reactivity. At sea-level and upon arrival at altitude, participants rested in seated position on a cycle ergometer before performing a VOT consisting of 5 min of tissue ischemia via femoral artery occlusion, followed by 5 min of vascular reperfusion[15]. To ensure signal stability, tissue ischemia was only initiated after tissue saturation index (TSI) had been stable for 30 s (< 2% variation). Occlusion was accomplished using a pneumatic cuff placed on the proximal part of the thigh and connected to an automatic rapid inflation system (HokansonE20 AG101, Bellevue, WA, USA). Occlusion pressure was set between 290 and 300 mmHg and maintained for the full 5-min period of ischemia. Cuff pressure was identical at sea-level and at altitude. Oxygenation changes in the *vastus lateralis* muscle were evaluated by a continuous wave NIRS device (Portamon, Artinis Medical Systems, Elst, The Netherlands), which consisted of three dual-wavelength (760 and 850 nm) light transmitters-channels and one receiving optode. Three distances (30, 35 and 40 mm) were adopted between the receiver and transmitters. The NIRS probe was placed on the lower third of the *vastus lateralis* muscle (~ 10 cm above the knee joint) of the right thigh. The skin overlying the investigated muscle region was

carefully shaved before experimentation, and the same experimenter placed the probe to minimize the variability in positioning between tests. The probe was secured to the skin using double-sided tape, and elastic bandages were wrapped around the probe and the thigh to avoid ambient light contamination. Adipose tissue thickness (ATT) at the site of the NIRS probe was measured using a skinfold caliper. TSI changes were monitored during the test. NIRS data were recorded at 10 Hz and exported at 5 Hz. Baseline TSI (%) was calculated as the 30 s average before cuff occlusion. As previously proposed[15,21], the linear regression of TSI signal during the first minute of occlusion (desaturation rate, % s^{-1}) was taken as an index of resting skeletal muscle oxidative metabolism. The rate of reperfusion (reperfusion rate, % s^{-1}) was quantified as the upslope of the TSI signal during the first 10 s following cuff release. Minimum and peak TSI (TSI_{min} and TSI_{peak}, respectively, %) were calculated as the lowest and highest recorded TSI value during ischemia and reperfusion, respectively, and their difference was used to compute the amplitude of the change (A_{TSI}, %). The time required for the TSI signal to return to baseline values after cuff release ($t_{baseline}$) was also evaluated. The area under the curve for the TSI signal (AUC_{2MIN}) was calculated from the reperfusion overshoot (area under the reperfusion curve above baseline until 2-min post cuff release).

Skeletal muscle oxidative capacity. After having checked that the TSI signal following VOT returned to baseline values (~ 5 min), skeletal muscle oxidative capacity was evaluated in vivo by measuring post-exercise muscle oxygen consumption (mVO_2) recovery kinetics by NIRS and the repeated occlusions method[16,17]. The protocol consisted of a 3-min rest followed by a 10-min moderate intensity (~ 50% VO_{2peak}) constant work rate exercise bout aiming to increase mVO_2 and desaturate the muscle to ~ 50% of A_{TSI} (i.e. the physiological range)[18,22]. The exercise bout was immediately followed by a series of 5-s intermittent arterial occlusions (5 separated by a 5-s cuff release, 5 separated by a 10-s release, and 5 separated by a 20-s release). As per VOT, occlusion pressure was set between 290 and 300 mmHg and it was kept the same between the two conditions. For each intermittent arterial occlusion, the rate of decline in TSI (expressed in % s^{-1}) was fitted by a linear function to estimate relative mVO_2. Importantly, as previously reported[22], during arterial occlusion the deoxygenation rate is inversely proportional to mVO_2, and it is therefore reported below as a positive value (%s^{-1}). mVO_2 values were then fitted by a monoexponential function of the type:

$$y(t) = y_{END} - Delta \times e^{-1/\tau}$$

where, y(t) represents the mVO_2 value at a given time (t), y_{END} the mVO_2 value immediately after exercise cessation, Delta is the change in mVO_2 from rest to the end of exercise, and τ is the rate constant ($k = [1/\tau]$, expressed in min^{-1}) of the function. The exponential mVO_2 recovery rate constant (k, min^{-1}) was estimated using nonlinear least-squares regression and was taken as an estimate of skeletal muscle oxidative capacity. As previously proposed[18,24], DmO_2 was estimated as the absolute difference between k obtained under normoxic and hypoxic conditions.

Plasma pro-oxidant/antioxidant balance. *Blood sampling.* At sea-level and upon arrival at altitude, 6 mL of venous blood were obtained from the antecubital vein with the participants in a seated position. Blood samples were drawn into ethylenediaminetetraacetic acid blood collection tubes and centrifuged (10 min at 3500 rpm, 4 °C). Subsequently, the obtained plasma was aliquoted into three 1.5 mL cryotubes, which were immediately frozen to −80 °C until analysis[25].

Biochemical analyses. All spectrophotometry and fluorometry measurements were performed with the TECAN Infinite 2000 plate reader (Männedorf, Switzerland).

Oxidative stress markers. AOPP levels were measured via spectrophotometry by reading at 340 nm 40 µL of plasma diluted in 200 µL of PBS 1X with 20 µL of acetic acid (99–100%) in 96-well microtest plates. AOPP level was computed using chloramine-T standard solution, which absorbs at 340 nm in the presence of potassium iodide.

Xanthine oxidase (XO) activity was determined by measuring the appearance kinetics of the complex superoxide anion and nitrotetrazolium blue (NTB) by spectrophotometer at 560 nm for 10 min.

Similarly, myeloperoxidase (MPO) activity was measured by a semi-quantitative immunoassay using stabilized human anti-MPO antibodies (MPO, Human, clone 266-6K1, HM2164, Hycult Biotech). The MPO/anti-MPO complex was detected by spectrophotometry after addition of a 3,3',5,5'-tetramethylbenzidine solution (TMB, Sigma) with H_2O_2 as a chromogenic substrate.

Antioxidant enzymes. Catalase activity was determined by measuring the kinetics of formaldehyde apparition formed by the reaction between methanol and hydrogen peroxide (H_2O_2), which is catalyzed by catalase. 30 µL of methanol (100%) and 20 µL of H_2O_2 solution (0.14%) were added to 20 µL of plasma diluted in 100 µL of PBS 1X in 96-well microtest plates. After 20 min the reaction was inhibited by adding 30 µL of a potassium hydroxide solution (10.69 mol·L^{-1}). Formaldehyde was revealed by adding 30 µL of purpald solution (0.20 mol·L^{-1}), and its concentration was measured 5 min later by spectrophotometry at 540 nm and computed using formaldehyde standards.

GPx activity was assessed by measuring nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) consumption, which is proportional to GPx activity to reduce H_2O_2 in the presence of glutathione reductase and reduced glutathione. Glutathione reductase, NADPH (10 mmol·L^{-1}) and reduced glutathione solutions

	Term born	Preterm	*P*-value
Participants' characteristics			
Gestational age (weeks)	40 ± 0	29 ± 1	$P < 0.001$
Birth weight (g)	3621 ± 101	1132 ± 64	$P < 0.001$
Age (years)	21 ± 1	21 ± 1	$P = 0.490$
Height (cm)	182 ± 2	178 ± 2	$P = 0.210$
Body mass (kg)	75.6 ± 1.7	72.4 ± 3.5	$P = 0.415$
Body mass index ($kg \cdot m^{-2}$)	22.8 ± 0.4	22.5 ± 0.7	$P = 0.713$
Body surface area (m^2)	1.95 ± 0.03	1.89 ± 0.06	$P = 0.322$
VO_{2peak} ($mL \cdot min^{-1} \cdot kg^{-1}$)	51.9 ± 1.9	48.5 ± 2.6	$P = 0.290$

Table 1. Participants' physical characteristics (Mean ± SEM).

were added to 20 µL of plasma diluted in 200 µL of PBS 1X in 96-well microtest plates. 30 µL of H_2O_2 solution was then added and NADPH oxidation into NAD^+ was measured for 5 min by spectrophotometry at 340 nm.

Total SOD activity measurement was based on the higher affinity of SOD for superoxide anion ($O_2^{\bullet -}$) than nitrobluetretrazolium (NTB), producing detectable blue formazan. 250 µL of a solution containing NTB, trizmahydrochloride, diethylenetriaminepentaacetic acid and hypoxanthine was added to 20 µL of plasma in 96-well microtest plates. 20 µL of xanthine oxidase ($1.02 U \cdot mL^{-1}$) was then added and reacted with hypoxantine to produce $O_2^{\bullet -}$. The appearance of blue formazan was measured by spectrophotometry at 560 nm for 5 min. SOD activity was computed by subtracting the rate of blue formazan appearance with deproteinized plasma (blank) to those with plasma sample.

Nitric oxide (NO) metabolites. Nitrite (NO_2^-) levels were detected by using 2,3-diaminonaphtalene (DAN) which fixes nitrite and emits at 450 nm after an excitation at 365 nm. 18 µL of DAN solution containing DAN and HCl was added to 10 µL of plasma diluted in 90 µL of H_2O in 96-well microtest plates. After 10 min the reaction was inhibited with 18 µL oh NaOH solution. NO_2- levels was measured by fluorometry (excitation at 365 nm and emission at 450 nm) and computed with NO_2 standards.

To measure total NO_2- and nitrate (NOx) levels, nitrate was reduced into NO_2-, and NO_2- was then measured as described above. 40 µL of nitrate reductase solution was added to 10 µL of plasma in 96 well microplates and 15 min later 50 µL of H_2O was added. Then NO_2- level is determined as described above.

Statistical analyses. The required sample size of fifteen preterm and fifteen term born adults was established a priori ($\alpha = 0.05$, $\beta = 0.80$) based on previous data from others[21] and by our research group[25] investigating microvascular reactivity and oxidative stress responses, respectively, in different populations.

All data are presented as mean ± SEM throughout the manuscript. A two-way (group × condition) repeated measures ANOVA was performed to compare changes in microvascular and oxidative stress responses during acute exposure to altitude between preterm and term born adults. Significant interaction effects were analyzed by Sidak correction. After having checked for normality using the Shapiro–Wilk normality test, pulmonary function, and percent changes from sea-level to high-altitude in preterm and term born individuals were compared by independent student's t-test. Linear regression and correlation analyses were carried out by the least-squared residuals method. All *p*-values are two-tailed and statistical significance was defined a priori at $p < 0.05$. Data analyses were performed using the statistical software package Prism v.6.0 (GraphPad Software, San Diego, CA, USA).

Results

Participants' physical characteristics are reported in Table 1. Participants' spirometry and lung diffusion capacity to carbon monoxide were presented in Manferdelli et al.[4]. No difference was observed between the two groups.

Systolic blood pressure was similar between groups (preterm: 128 ± 2 mmHg; term born: 127 ± 2 mmHg, $p = 0.633$) and conditions (sea-level: 128 ± 2 mmHg; high-altitude: 127 ± 2 mmHg, $p = 0.571$), while a main effect of condition was found for diastolic blood pressure (sea-level: 75 ± 2 mmHg; high-altitude: 79 ± 1 mmHg, $p < 0.001$).

At sea-level, ATT at the site of the NIRS probe was similar between prematurely born (4.8 ± 0.6 mm) and term born (4.8 ± 0.4 mm, $p = 0.983$) adults and it was not affected by acute exposure to high-altitude in both preterm (4.8 ± 0.5, $p = 0.797$) and term born (4.5 ± 0.4, $p = 0.122$) participants.

NIRS-VOT-derived parameters of muscle oxygenation and reactivity. Average TSI dynamics for both groups at sea-level and at high-altitude are shown in Fig. 1A, B, respectively. NIRS-VOT-derived parameters are reported in Fig. 2. Interestingly, the reperfusion rate did not change from sea-level to high-altitude in preterm (1.54 ± 0.15% s^{-1} vs. 1.57 ± 0.14% s^{-1}, $p = 0.969$) participants while it increased in term born (1.48 ± 0.11% s^{-1} vs. 1.83 ± 0.12% s^{-1}, $p = 0.008$) adults. Similarly, $t_{baseline}$ was similar at high-altitude compared to sea-level in preterm (13.6 ± 0.9 s vs. 14.7 ± 1.5 s, $p = 0.539$) adults, while it was faster in term born (14.2 ± 1.0 s vs. 11.0 ± 0.7 s,

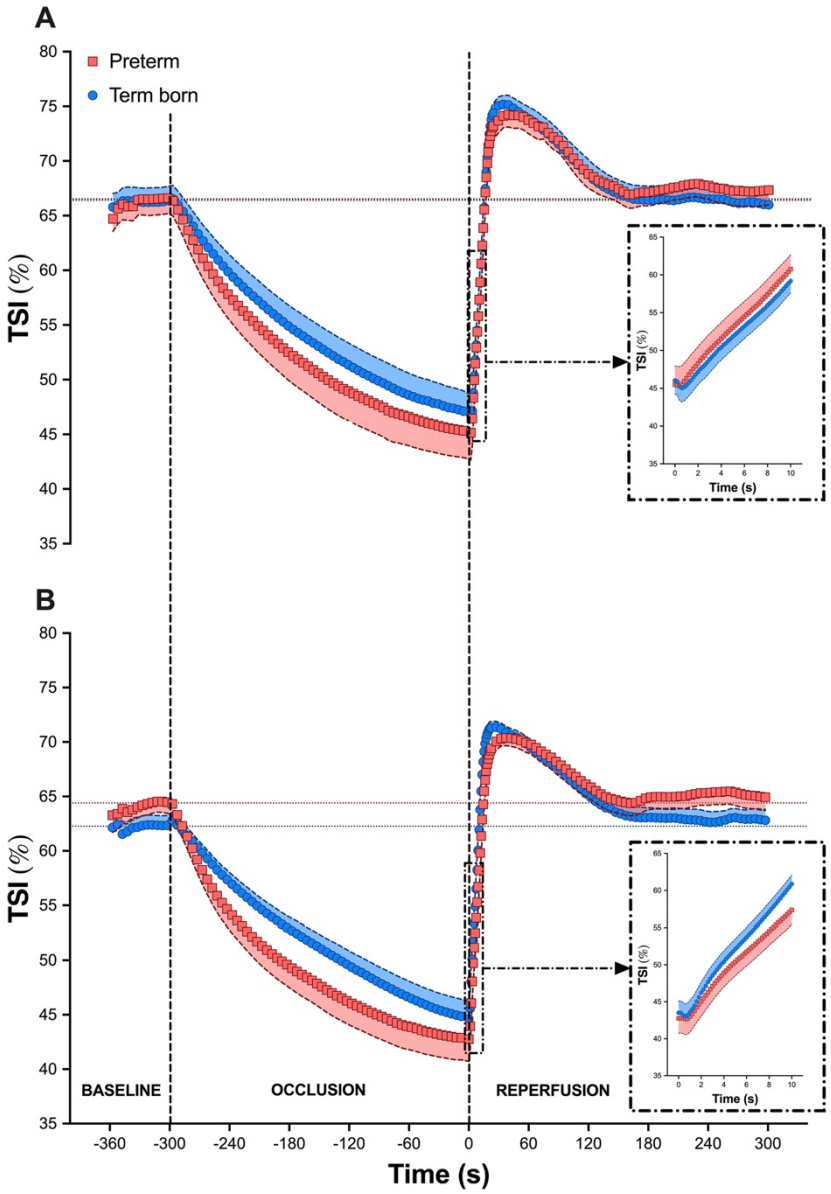

Figure 1. Groups mean (±SEM) of oxygen saturation signal (TSI) during the vascular occlusion test (VOT) performed at sea-level (panel **A**) and during acute exposure to high-altitude (3375 m; panel **B**) in prematurely born (red squares) and term born (blue circles) adults. Vertical dashed lines indicate instances of cuff inflation and deflation, while horizontal dashed lines indicate baseline values for each group. For each condition, the lower panel on the right side depicts the mean data during the first 10 s after cuff release (reperfusion rate).

$p = 0.008$) participants. In turn, $t_{baseline}$ *was* significantly slower in preterm compared to term born participants upon acute high-altitude exposure ($p = 0.035$).

Skeletal muscle oxidative capacity. A graphical representation of TSI changes during the repeated arterial occlusion protocol in a typical participant (*term born, normoxia*) is shown in Fig. 3. A similar k was found between groups both at sea-level ($p = 0.795$) and during acute exposure to high-altitude ($p = 0.625$; Fig. 4). DmO_2 was significantly lower in preterm compared to term born participants (-0.3 ± 0.2 min^{-1} vs. 0.4 ± 0.1 min^{-1}, $p = 0.029$).

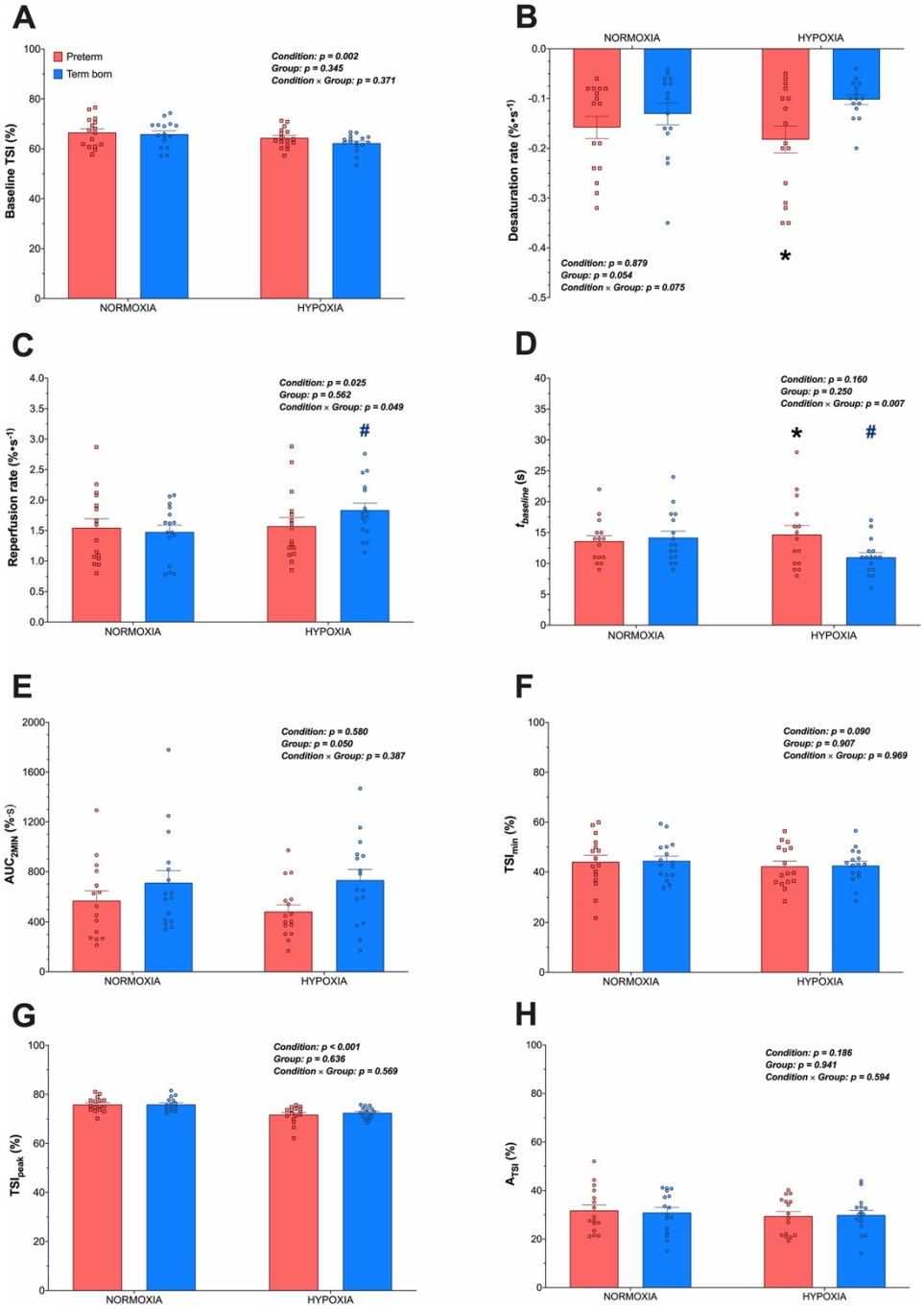

Figure 2. NIRS-VOT derived parameters (*panel* ***A***, baseline TSI; *panel* ***B***, desaturation rate; *panel* ***C***, reperfusion rate; *panel* ***D***, time to baseline ($t_{baseline}$); *panel* ***E***, area under the curve above baseline value during the first 2 min after cuff release (AUC_{2MIN}); *panel* ***F***, minimum TSI value reached during the occlusion phase (TSI_{min}); *panel* ***G***, peak TSI value reached during the occlusion phase (TSI_{peak}); *panel* ***H***, amplitude of TSI change during the reperfusion phase (A_{TSI}) in prematurely born (red squares) and term born (blue circles) participants at sealevel and during acute exposure to altitude (3375 m). Data are expressed as Mean ± SEM. *Significantly different from term born; #Significantly different from normoxia.

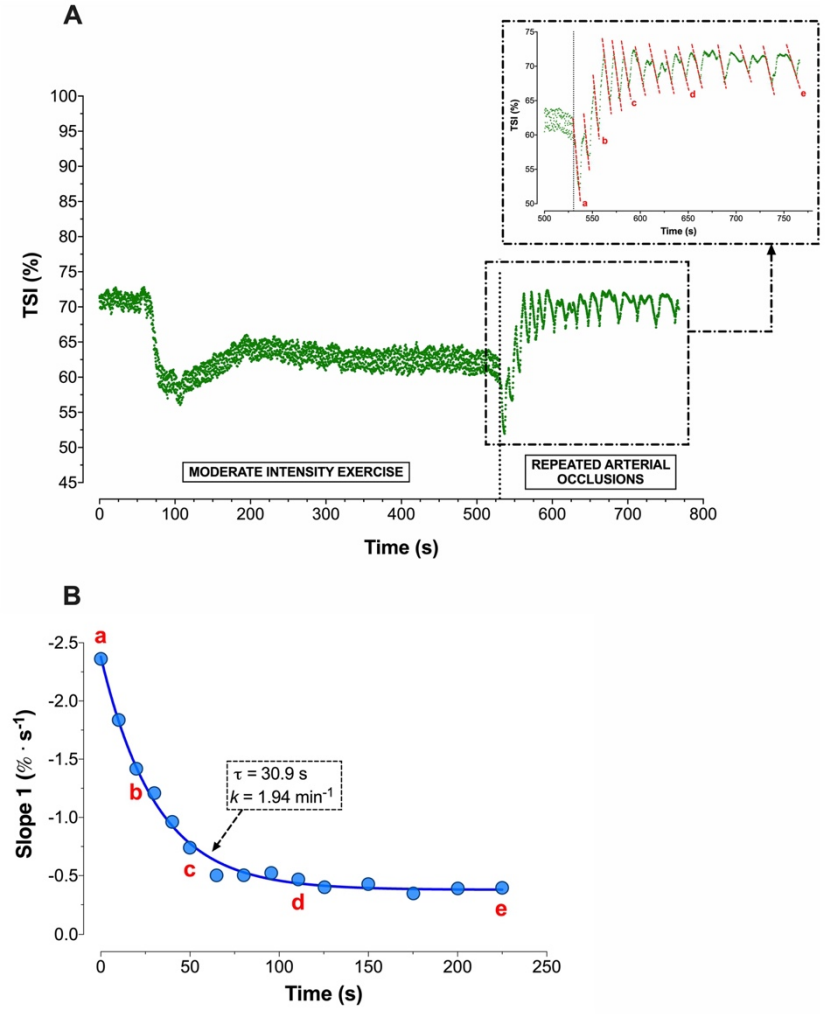

Figure 3. Representative participant (*term born, normoxia*) response during the muscle oxidative capacity assessment. Panel (**A**) illustrates the TSI dynamics during moderate intensity exercise followed by a series of intermittent arterial occlusions during recovery. The panel on the upper right corner shows the TSI changes during intermittent arterial occlusions and the red dotted lines represent the linear regression during each occlusion. Panel (**B**) depicts the slopes of each occlusion and the calculated muscle VO_2 (mVO_2) recovery profiles (dashed line). *k* represents the rate constant, which is linearly related to muscle oxidative capacity ($k = (1/\tau)$ 60, min^{-1}. The letters (a–e) illustrate how the corresponding mVO_2 value is derived from respective TSI negative slopes during intermittent occlusions.

Oxidative stress. Oxidative stress markers, antioxidant enzyme and NO metabolites at sea-level and at high-altitude in both groups are reported in Fig. 5. The altitude-induced increase in plasma XO was significantly lower in preterm compared to term born participants (28.7 ± 26.0% vs. 159.3 ± 48.7%, $p = 0.030$). In contrast, preterm adults showed a more pronounced altitude-induced increase in plasma AOPP and catalase (35.3 ± 61.0% and 66.9 ± 63.9%, respectively) compared to term born adults (-13.2 ± 13.2% and 15.2 ± 16.9%, $p = 0.034$ and $p = 0.010$, respectively).

Correlations. At sea-level, the reperfusion rate was significantly correlated with $t_{baseline}$ in both preterm ($r = -0.591$, $p = 0.020$) and term born ($r = -0.567$, $p = 0.022$) adults. Likewise, $t_{baseline}$ was significantly correlated with NO_2^- levels in preterm ($r = -0.559$, $p = 0.038$) but not term born participants ($p = 0.135$). Moreover, diastolic blood pressure was correlated with the reperfusion rate in preterm ($r = 0.611$, $p = 0.012$), but not in term born participants ($p = 0.588$).

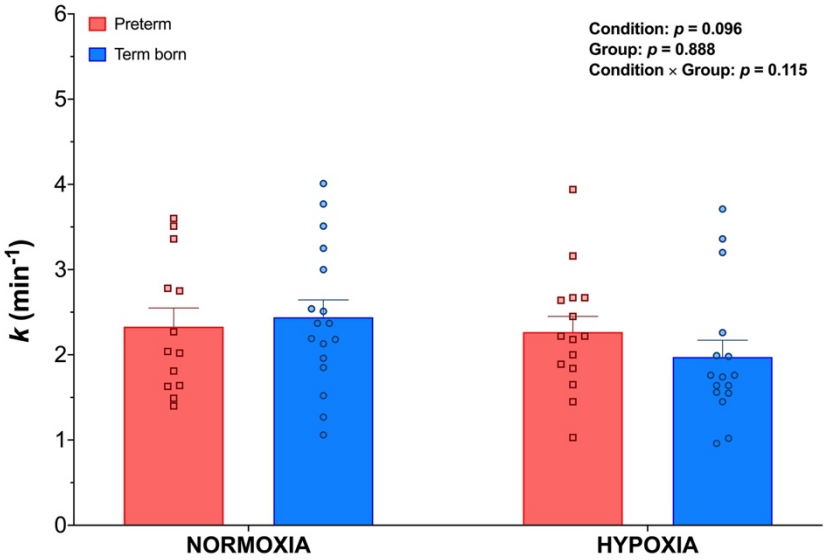

Figure 4. Groups mean (±SEM) of skeletal muscle VO_2 recovery rate constant (k) at sea-level and during acute exposure to high-altitude (3375 m) in prematurely born (red squares) adults and their term born (blue circles) peers. *Significantly different from term born.

Discussion

The aim of this study was to investigate the peripheral (microvascular reactivity and skeletal muscle oxidative capacity) and systemic oxidative stress responses to acute high-altitude exposure in prematurely born but otherwise healthy adults and in their term born peers. To the best of our knowledge, this is the first study to report a blunted post-occlusive reactivity at the microvascular level and higher skeletal muscle oxidative capacity during acute exposure to high-altitude in adults born preterm compared to term born participants. Also, prematurity appeared to confer specific altitude-induced changes in some pro-oxidant/antioxidant markers.

The microvascular responsiveness was assessed by monitoring NIRS-derived TSI changes in the *vastus lateralis* muscle during VOT[14,15]. Under normoxic conditions, a blunted microvascular reactivity to a 5-min arterial occlusion was observed in patients with cardiovascular diseases[19] and sedentary individuals[20,21] compared to healthy and trained subjects, respectively. In addition, microvascular reactivity assessed by NIRS in the thenar muscles at different altitudes was reduced following a short period of ischemia (3 min) in resting skeletal muscle of acclimatizing healthy adults compared to sea-level[26]. In the present study, partly supporting our second hypothesis, acute high-altitude exposure did not induce any change in reperfusion rate or muscle oxygenation $t_{baseline}$ in preterm adults, while these responses were altered in term born individuals. Accumulating evidence suggests that acute exposure to hypoxia induces release of pro-angiogenic factors and local vasodilators (i.e., NO, adenosine, acidosis), leading to capillary recruitment and increased microcirculatory oxygen extraction capacity[27]. It is therefore reasonable to speculate that skeletal muscle microvascular responsiveness was improved in term born individuals, likely due to an increased number of perfused capillaries during acute exposure to altitude. In contrast, the microvascular response was unchanged in preterm individuals, suggesting a blunted microcirculation response to acute exposure to high-altitude in this group. Our findings may therefore support previous results on lower microvascular function and density in healthy adults born prematurely[3]. Ultimately, this impaired physiological response may mechanistically underpin the potentially increased susceptibility of these individuals to high-altitude illnesses, in particular to high-altitude pulmonary and cerebral edema[28]. An important methodological point when assessing vascular responsiveness to VOT is the potential influence of the ischemic stimulus on the magnitude of the response (reperfusion)[14]. Given that high-altitude exposure decreased baseline TSI similarly in both groups (~4%), and that both term born and preterm participants reached similar TSI values at the end of the occlusion period, the differences observed in post-occlusive reperfusion in this study are unlikely to be due to a different ischemic stimulus.

The present study also investigated skeletal muscle oxidative capacity and DmO_2 using NIRS-derived TSI during the repeated occlusions method[16]. Even though acute hypoxia is known to impair mitochondrial function[10], the altitude-induced decrease in oxidative capacity was significantly lower in our preterm cohort compared to their term born peers. Increased mitochondrial oxygen consumption was observed in peripheral blood mononuclear cells of prematurely born adults[13]. In contrast, postnatal hyperoxic exposure in male rats was shown to reduce mitochondrial function relative to a control group provided with no treatment[12]. Despite these recent findings, oxidative function at the skeletal muscle level remains significantly under-investigated in this population. In fact, several comorbidities typically described in adult survivors of premature birth are characterized by increased mitochondrial dysfunction and chronic oxidative stress in non-preterm populations. To the best of

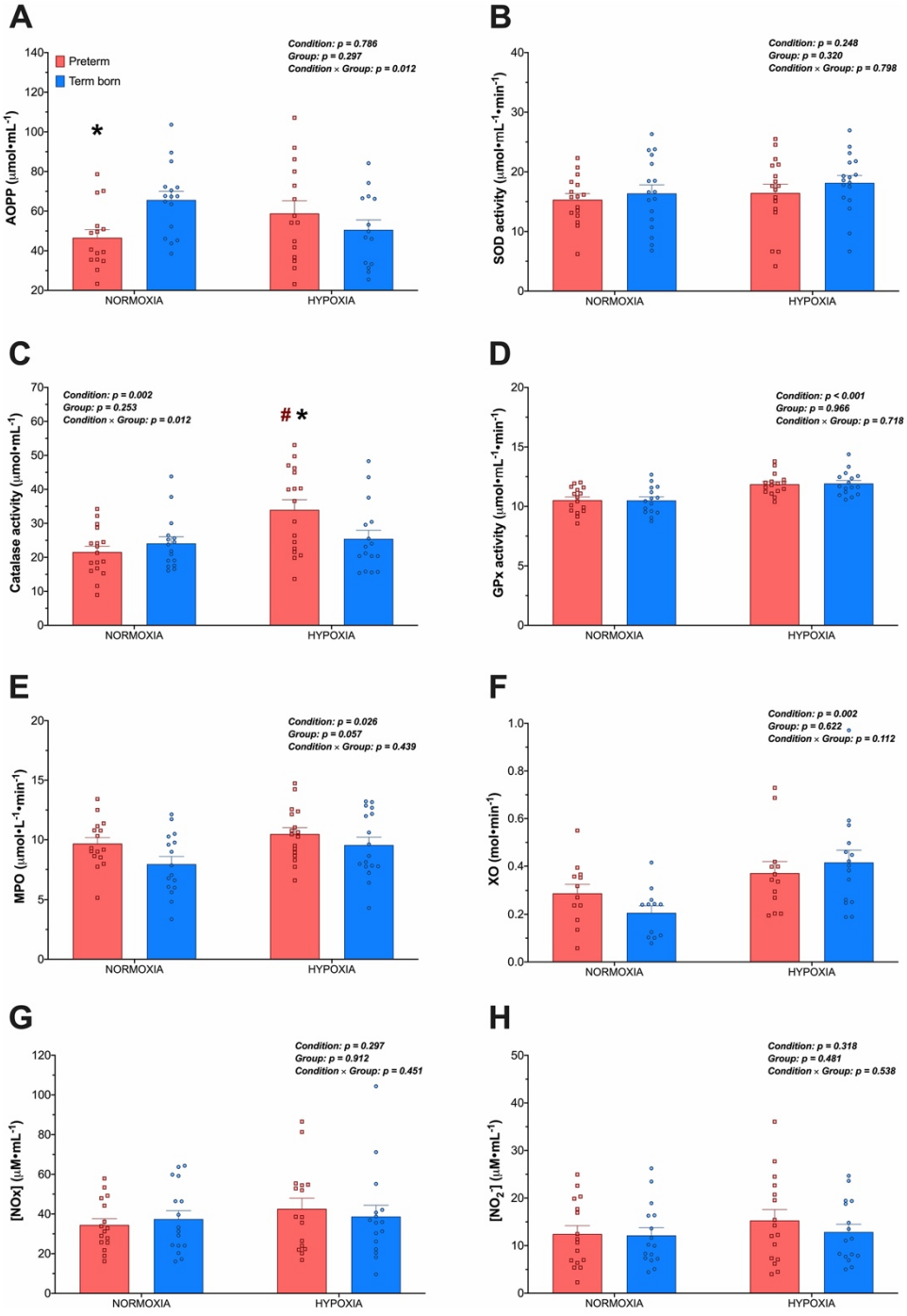

Figure 5. Groups mean (±SEM) of oxidative stress markers, antioxidant enzymes, and nitric oxide metabolites in plasma at sea-level and upon arrival at high-altitude (3375 m) in preterm (red squares) and term born (blue circles) adults. AOPP, advanced protein oxidation products (panel **A**); SOD, superoxide dismutase (panel **B**); catalase activity (panel **C**); GPx, glutathione peroxidase (panel **D**); MPO, Myeloperoxidase (panel **E**); XO, xanthine oxidase (panel **F**); NOx, total nitrite and nitrate (panel **G**); NO_2^-, nitrite (panel **H**). *Significantly different from term born; #Significantly different from normoxia.

our knowledge, we are the first to report a lower susceptibility to altitude-induced decreases in skeletal muscle oxidative capacity in preterm adults. However, further research is warranted to elucidate the underlying mechanisms of this specific response to acute high-altitude/hypoxia in this cohort.

Furthermore, DmO_2 assessed as recently proposed[18,24] was lower in preterm adults compared to their born term peers. Although influenced by many other factors, DmO_2 is proportional to capillary density[29], and represents one of the key determinants of exercise capacity in humans[30]. However, a recent study published by our group demonstrated that exercise capacity in preterm adults—with similar relative VO_{2peak} but lower peak power output compared to term born peers—is primarily impaired by convective rather than diffusive O_2 transport mechanisms[4]. Despite this, peripheral skeletal muscle limitations have recently been suggested to be an important underlying mechanism limiting exercise capacity in adults born preterm[31]. The present findings of reduced DmO_2 are in line with previous results from animal models[12] and suggest skeletal muscle specific long-term prematurity sequelae.

Finally, exposure to hypoxia increases oxidative stress in the general term born population[25]. The present study also investigated NO metabolites and oxidative stress markers in response to acute exposure to high-altitude. Increased oxidative stress in normoxic conditions was reported in preterm infants due to their immature defense systems against reactive oxygen species (ROS), immature organs and the need for medical treatments to increase ROS production[10]. In this population, higher oxidative stress levels persist through young adulthood and the subsequent redox imbalance might result in a 'preconditioning' state, or it may be involved in the pathogenesis of several non-communicable chronic diseases[10]. However, the present study did not find an increased oxidative stress in normoxia in prematurely born adults. Our contrasting results might be explained by the relatively high VO_{2peak} of our preterm cohort compared to the values typically reported in the literature for this population[32]. However, a lower altitude-induced increase in XO and higher levels of both AOPP and catalase in response to acute high-altitude exposure were observed in preterm compared to term born participants, suggesting an increased systemic H_2O_2 production upon arrival to high-altitude in preterm compared to term born adults. Likewise, the lower hypoxia-induced increase in XO during acute high-altitude exposure in prematurely born adults may be an adaptive mechanism in addition to the increased catalase activity. A recent comprehensive review on the adaptive mechanisms to hypoxic exposure highlighted mitochondria and activation of nicotinamide adenine dinucleotide phosphate oxidase as main sources of ROS formation in humans[33]. Although systemic oxidative stress may not be reflective of solely muscle oxidative stress only, skeletal muscles represent the main source of oxidative stress, particularly in under combined hypoxia and exercise[34,35]. Therefore, it could be hypothesized that a greater skeletal muscle oxidative capacity should result from an upregulated mitochondrial function. In turn, the exaggerated mitochondrial function in preterm recently reported in this population[13], could lead to an even greater release of reactive oxygen species from the muscle and explain their increased systemic oxidative stress. Therefore, the increased ROS formation in this study, in conjunction with higher skeletal muscle oxidative capacity during acute high-altitude exposure, suggest an upregulated mitochondrial function in preterm individuals compared to their term born peers under conditions of reduced oxygen availability. In contrast, NOx and NO_2^- were not affected by exposure to high-altitude in both groups. While NO is undoubtedly involved in the pulmonary, cardiovascular, and muscular responses to high-altitude exposure[36], a recent well-conducted study demonstrated that NO metabolites peak after 3 days at an altitude similar to that used in the present study. Furthermore, the present study did not observe a direct correlation between plasma NO_2^- and NOx concentrations and microvascular responsiveness at sea-level in term born adults. Despite the central role of NO on both macro and microvascular function[37], it seems that other active signaling molecules and/or patterns (e.g., PGI_2, Adenosine, K_{ATP} channels) regulated by tissue hypoxia concomitantly regulate vasodilation processes and muscular perfusion[14]. On the contrary, $t_{baseline}$ was inversely correlated with NO_2^- levels in prematurely born participants, suggesting that microvascular function in this population is primarily driven by NO metabolites at sea-level.

Methodological considerations. Even though the present study was the first to assess microvascular responsiveness and in vivo skeletal muscle oxidative capacity in prematurely born adults at both sea-level and acute high-altitude exposure, we would like to acknowledge a few important limitations. First, our prematurely born cohort had a relatively high VO_{2peak} and normal pulmonary function compared with those generally reported by others[32]. Therefore, the relatively active preterm adults investigated in this study may not ideally represent typical preterm born adults. However, according to recent findings[38], this might be a consequence of them being born at the turn of the millennium with better neonatal management and consequently improved long-term pulmonary outcomes. Given the continued advancements in neonatal medicine, studies of preterm birth survivors receiving more contemporary treatments are (and will continue to be) of increasing importance. In addition, the present study investigated only male survivors of preterm birth, despite existing evidence in rodent studies that sex differences likely exist in the association between preterm birth and skeletal muscle physiology[12]. We acknowledge the need for further research on the long-term sequelae of premature birth in female participants. Finally, one may question whether the compensatory mechanisms observed in this study were maximised in the preterm born group. Unfortunately, the present study was unable to quantify this response in relation to the individual capacity to adapt to high altitude. This could however be a particularly interesting avenue for future research.

What is the clinical relevance of the present findings? A growing body of literature suggests a specific phenotypical cardiovascular response to hypoxia in this cohort[4,32,39]. While some authors hypothesized an increased risk of right ventricular failure due to an exaggerated cardiac contractile response to hypoxia, our findings seem to suggest an unresponsiveness of the microcirculation to acute exposure to high-altitude in preterm adults. Intriguingly, a blunted microvascular responsiveness was suggested to represent an early sign for later

macrovascular dysfunction[40]. It seems therefore that hypoxia may represents a risk factor for the cardiovascular system in this population. However, we recommend further studies before drawing conclusions of clinical relevance.

Conclusion

Overall, this study provides novel insights into the peripheral (microvascular responsiveness and skeletal muscle oxidative capacity) and oxidative stress responses to acute high-altitude exposure in adults born prematurely. Preterm adults showed higher oxidative stress and blunted peripheral responses to acute exposure to high-altitude compared to term born participants, as demonstrated by unchanged microvascular reactivity and skeletal muscle oxidative capacity. This observed blunted response to acute high-altitude exposure observed in prematurely born (but otherwise healthy) adults may compromise altitude acclimatization and ultimately lead to an increased risk of high-altitude illnesses. Future research is necessary to substantiate our findings and should focus on measuring skeletal muscle diffusion and oxidative capacity using direct methods both at rest and during exercise, as well as under different environmental conditions such as hypoxia or hypobaria.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
G.P.M. and T.D. conceived the research and obtained the financial support. G.M., B.J.N., G.G., T.D., and G.P.M. contributed to the experimental design. G.M. and B.J.N. collected the data. V.P. analyzed the plasma samples for oxidative stress markers, antioxidant enzymes, and nitric oxide metabolites. G.M., V.P., and G.P.M. analyzed and interpreted the data. G.M. drafted the manuscript. All authors critically revised the draft and approved the final version of the manuscript.

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Competing interests
The authors declare no competing interests.

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11. Article 3

Changes in baroreflex sensitivity differ in hypoxia but not in hypercapnia between healthy preterm and term born adults

Article 3 – Changes in baroreflex sensitivity differ in hypoxia but not in hypercapnia between healthy preterm and term born adults

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Changes in baroreflex sensitivity differ in hypoxia but not in hypercapnia between healthy preterm and term born adults

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Abstract

Premature birth confers specific cardiovascular and ventilatory responses to hypoxia and hypercapnia, leading to an increased risk of cardiovascular events at high-altitude. This study aimed to investigate the effects of premature birth, when exposed to hypoxia, hypobaria and hypercapnia on baroreflex sensitivity (BRS). At both sea-level and after 1 day at high-altitude (3375 m), BRS was assessed in seventeen young preterm born males (gestational age, 29 ± 1 weeks), and seventeen age-matched term born adults (40 ± 0 weeks) during consecutive 6-min stages breathing different concentrations of oxygen and carbon dioxide. Continuous blood pressure and ventilatory gas exchanges were recorded in normobaric normoxia (NNx), normobaric normoxic hypercapnia (NNx+CO_2), hypobaric hypoxia (HHx), hypobaric normoxia (HNx), hypobaric normoxia normocapnia (HNx+CO_2), and hypobaric hypoxia with end-tidal CO_2 clamped at the same NNx value (HHx+clamp). BRS was assessed using the sequence method. Across all conditions, BRS was lower in term born compared to preterm (13.0 ± 7.5 vs. 21.2 ± 8.8 ms·mmHg^{-1}, main group effect: $p < 0.01$) participants. BRS was lower in HHx compared to NNx in term born (10.5 ± 4.9 vs. 16.0 ± 6.0 ms·mmHg^{-1}, $p = 0.05$), but not in preterm (27.3 ± 15.7 vs. 17.6 ± 8.3 ms·mmHg^{-1}, $p = 0.43$) participants, leading to a lower BRS in HHx in term born compared to preterm ($p < 0.01$) adults. In conclusion, this study reports a blunted resetting of BRS during acute high-altitude exposure in healthy prematurely born adults.

NEW & NOTEWORTHY

This study supports previous finding on specific cardiovascular responses to hypoxia in prematurely born adults and extends those to a blunted hypoxia-induced decrease in spontaneous cardiovagal baroreflex sensitivity at high-altitude. However, the baroreflex response is not impaired by breathing additional CO_2, either in normoxia or hypoxia, in this cohort compared to control participants born at term.

Altogether, this study shows a blunted BRS in hypoxia but no changes in hypercapnia in preterm adults.

Key words: baroreflex, altitude, hypercapnia, preterm, hypobaria

Introduction

Premature birth (< 37th week of gestation) is a growing health concern due to the increasing number of preterm survivors reaching adulthood reporting several prematurity-related long-term sequelae on the cardiovascular and respiratory systems (14, 26). Mounting evidence reports systemic and pulmonary artery hypertension in young adult survivors of premature birth (1, 9) leading to an increased risk of cardiovascular diseases and events during adulthood (27, 33). Even though human investigations and animal models on the mechanisms by which premature birth *per se* leads to increased blood pressure (BP) are very limited, a recent work demonstrated impaired vascular structure and function in young adults born preterm with high systolic blood pressure (SBP) in adult life (25).

The autonomic nervous system (ANS) has been extensively implicated in the onset of several cardiovascular diseases (5, 28) and growing evidence indicates reduced vagal activity, and thus relative sympathetic dominance (i.e. autonomic imbalance), as a potential pathway to increased morbidity and mortality from cardiovascular disease (50). Limited results also suggest autonomic dysfunction in adult survivors of premature birth (19, 22, 36) and therefore raise questions about its contribution in the higher risk of cardiac diseases in this population.

Baroreflex sensitivity (BRS) is typically used to investigate the integrated function of the autonomic nervous and cardiovascular systems in humans (23). The baroreflex is a powerful BP regulatory mechanism that detects changes in BP and evokes reflex circulatory adjustments aiming to buffer BP changes (24). The sensitivity of the arterial baroreflex control or cardiac activity plays a pivotal role in human health as demonstrated by the inverse relation between BRS and the risk of mortality after myocardial infarction (23) or conversely by the improved BRS after endurance training (41).

High-altitude exposure is known to challenge both autonomic balance (18) and BP regulation in humans (7, 8), with recent evidence attributing a central role of the ANS in the acute response

and adaptation to hypobaric hypoxia (7, 8, 18). Acute hypoxia is a potent activator of sympathetic activity, leading to increased release of catecholamines, increases in heart rate and regional vasoconstriction (18). Meanwhile, baroreceptor afferents principally counteract the hypoxia-induced rise in BP by increasing/decreasing the activity of the parasympathetic and sympathetic branches of the ANS. Previous works demonstrated that spontaneous BRS decreases during acute and prolonged exposure to altitude (7, 8). This resetting of BRS is clear above 4500 m whilst is less evident for lower altitudes (46).

Increasing evidence demonstrates specific cardiovascular (2, 16, 38), respiratory (3, 11-13, 15, 38) and molecular (34, 35) responses to both hypoxia and hypercapnia in prematurely born children and young adults. While some authors reported beneficial effects of preterm birth during acute hypoxia exposure (15, 16), others suggested exaggerated and/or impaired responses to a reduced oxygen (O_2) availability (2, 3, 11) or increased CO_2 breathing (32, 47). The aim of the present study was therefore twofold: a) to compare the spontaneous cardiovagal BRS at sea-level and after 1 day of exposure to high-altitude in term born and prematurely born adults, and b) to investigate the effects of different combination of hypobaric, hypoxic and hypercapnic gas mixtures on spontaneous cardiovagal BRS in term born controls and prematurely born but otherwise healthy adults. We hypothesized first an impaired BRS response to hypoxic exposure and second a larger influence of hypercapnia on BRS in prematurely born adults compared to term born participants.

Methods

Participants

Seventeen term born and seventeen healthy preterm male participants volunteered and gave written informed consent to participate in this study. Participants were matched for age and for fitness status. All participants were not taking any medication and were free from any

cardiorespiratory, neurological and hematological diseases. Importantly, inclusion criteria for the preterm participants were postnatal hyperoxic treatment and that none of them have or had history of bronchopulmonary dysplasia. The experimental protocol was pre-registered at ClinicalTrials.gov (NCT04739904), approved by both the National Medical Ethics Committee of Slovenia (0120-101/2016-2) and the Aosta Hospital Ethical Committee, and it was performed according to the Declaration of Helsinki.

Experimental design and ascent protocol

Participants were tested in two different occasions (outlined below), one at sea level (295 m; Ljubljana, Slovenia; barometric pressure ~737 ± 2 mmHg) and the other at high-altitude (3375 m; Torino hut, Aosta Valley, Italy, on the Mont Blanc massif; barometric pressure ~503 ± 3 mmHg). Participants reached Torino hut from Courmayeur (1300 m) by cable car in about 15-20 minutes. At altitude, tests were performed in the first morning after arrival at altitude. Sea-level testing was performed between 2 and 3 months before ascending to altitude. Detailed description of the general experimental design of the project was previously published elsewhere (30).

Experimental protocol

Before each trial, the participants were instructed to abstain from exercise for 12 hours, avoid alcohol and caffeine for 24 hours, and they did not consume a heavy meal within 4 hours before testing. All experiments were conducted in the morning (9:00 – 11:00 a.m.) at the same time of the day with the participant comfortably seated on a chair. Following 10-15 min of quiet rest in seated position, each testing session included equipment instrumentation and six (two at sea-level and four at high-altitude) 6-min stages of continuous BP, HR, and gas exchanges recording in the following order: (1) normobaric normoxia (NNx), (2) normobaric normoxia hypercapnia (NNx+CO_2), (3) hypobaric hypoxia (HHx), (4) hypobaric normoxia (HNx), (5)

hypobaric normoxia hypercapnia (HNx+CO_2), (6) hypobaric hypoxia with $P_{ET}CO_2$ clamped at NNx value (HHx+clamp). Normobaric conditions were performed in Ljubljana, while hypobaric measurements were carried out at Torino hut. NNx+CO_2 was induced by switching the inspired gas from ambient air to 3% CO_2 (in 20.93% O_2, balance N_2). In HNx and HNx+CO_2, participants breathed supplemental O_2 (FiO_2=32%, with 0.03% CO_2, balance N_2 and with 3% CO_2, balance N_2, respectively). This O_2 concentration was calculated in order to induce the same inspired oxygen partial pressure that participants breathed during NNx. During condition 6, end-tidal clamping was performed using a modified version of the system developed by Olin, Dimmen, Subudhi and Roach (39). Briefly, the system is designed to deliver high-flow, low-resistance inspired gas with a fixed FiO_2 and a varying inspired fraction of CO_2 ($FiCO_2$). The inspiratory endpoint of this system included an open-ended reservoir where room air was mixed with 100% CO_2 compressed gas. The 8-L custom-made reservoir was connected, via a plastic flexible tube, to a 2-way non rebreathing valve (Hans Rudolph, 2700 series, Hans Rudolph, Kansas City, MO, USA) which was attached a low dead-space face mask (Hans Rudolph mask, 7400 oronasal series; dead space, 73 mL).

Measurements

Blood pressure and heart rate. Beat-to-beat systolic, diastolic, and mean arterial blood pressure (SBP, DBP, and MAP, respectively), as well as heart rate (HR), were monitored non-invasively using a finger photopletismography device (NIBP100D, Biopac Systems Inc., Goleta, CA, USA) combined to a double cuff installed on the index and the middle fingers. Automatic calibration of the device was performed immediately before the start of each test by measuring BP on the left arm of the participant at the level of the brachial artery using a cuff.

Respiratory gas exchange parameters. Participants breathed trough a leak-free respiratory mask (Hans-Rudolph 7450 series) attached to a T-shaped two-way non-rebreathing valve (see

Experimental protocol). $P_{ET}CO_2$, pulmonary ventilation ($\dot{V}_E$), tidal volume (V_T), and breathing frequency (B_f) were measured breath-by-breath and recorded using a metabolic cart (Ergocard Professional, Medisoft, Sorinnes, Belgium).

Arterialized Capillary Blood Gas variables. For each participant, capillary blood samples were taken from the earlobe during the last 30 s of each stage. Arterialization of capillary blood, aiming to induce a capillary shunting between arterial and venous territories, was achieved by applying a vasodilation cream (Capsolin, SIT s.r.l., Mede, Italy). Arterial blood gas parameters, including partial pressure of O_2 (P_aO_2), partial pressure of CO_2 (P_aCO_2), pH, hydrogen ion concentration ($[H^+]$), bicarbonate concentration ($[HCO_3^-]$), base excess, and arterial oxygen saturation (S_aO_2), were immediately analyzed using an arterial blood gas analyzer (ABL-90 FLEX, Radiometer, Copenhagen, Denmark). All samples were heated/corrected to an assumed resting body temperature of 37.0°C.

Acute Mountain Sickness (AMS). Symptoms of AMS were assessed in the morning of the test upon awake (about 16 hours after arrival at altitude) using the 2018 Lake Louise Scale (48). Accordingly, AMS was diagnosed when headache was present together with at least one additional symptom, and the total score was three or higher.

Data Acquisition

Cardiovascular data were measured continuously at 1000 Hz using an analog-to-digital converter (MP150, Biopac Systems Inc, Goleta, CA, USA, respectively). The device was interfaced to a PC using a dedicated software (Acknowledge v.4.2, Biopac Systems Inc, Goleta, CA, USA, respectively), and data were stored on computer for later off-line analyses. Signal processing was performed using custom Matlab routines (MATLAB, R2020b, MathWorks, Natick, MA, USA).

Data exclusion. Data from one subject were excluded from statistical analyses due to three fainting episodes (one in NNx and the others in HHx). Moreover, BP data from one participant at altitude were not available because of loss of signal from the photopletismography device. Therefore, overall, 16 term born and 17 preterm successfully completed stages 1-2, while 15 term born and 17 preterm completed stages 3-5. In addition, only 10 term born and 6 preterm were able to complete the 6 min of the during HHx+clamp phase.

Data Analyses

Respiratory variables for each condition were calculated as the average of the last 30 s of each stage. The hypercapnic ventilatory response (HCVR) at sea level (stages 1 and 2 of the experimental protocol) was calculated as the ratio between the delta in $\dot{V}_E$ and the delta in $P_{ET}CO_2$. SBP peaks were initially extracted from the BP waveform with heart beats representing the time of their occurrence and heart beat-to-beat time intervals (inter-beat interval, IBI) was extracted directly from BP recordings and calculated as the interval between successive systolic peaks. A second order polynomial was interpolated for each extracted peak using four neighbor samples (two immediately before and two immediately after) from the BP waveform in order to refine the heartbeats placement. Heartbeats were selected as the location of the maximum of the interpolated polynomial (8). Also, SBP values were updated as the maximum in their corresponding polynomial, and the IBI were created as the interval between successive peaks. BRS was then calculated using the sequence method, as previously described (8). Briefly, this method of analysis provides a direct interpretation of the causal link between BP and HR (40), and it was shown to be highly reliable (42). This method identifies at least three consecutive beats in which a strictly defined increase (or decrease) in SBP is followed by a strictly defined increase (or decrease) in the IBI. Fixed maximal changes were considered for SBP and IBI in order to consider the sequence valid. Specifically, a minimum change of 1 mmHg between two consecutive SBP peaks and or at least 5 ms change in a sequence (4). In

addition, to accept the sequence, the minimum correlation coefficient between changes in SBP and IBI was 0.85. finally, a minimum number of five sequences was set to validate a BRS estimate. The sensitivity of the baroreflex was obtained by computing the slope of the regression line between changes in SBP and IBI, and subsequently all the computed slopes were average to obtain the BRS. BRS was thus calculated using a 6-min window throughout each stage.

Statistical analysis

The number of recruited participants was established on *a priori* calculation ($\alpha = 0.05$, $\beta = 0.80$) based on previous works from our research group (7, 8) investigating BRS in the general population under the influence of different environmental stimuli (i.e. hypobaria, hypoxia, hypercapnia).

All data are presented as mean ± SD throughout the manuscript. Figure 1 display BRS, SBP, and HR, in Tukey boxplots in which the horizontal line inside the boxes is the median, whilst the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers denote the highest and lowest data points within the 1.5 inter quartile range which corresponds to approximately 2.7s and 99.3% coverage of the data.

A two-way (Group × Condition) repeated measures ANOVA was performed to compare BRS and respiratory gas exchange parameters between term born and preterm participants. Significant main and interaction effects were analyzed by Sidak correction. After having checked for normality, HCVR was analyzed by unpaired Student *t* test. AMS prevalence was analyzed by Fisher's exact test. All *p*-values are two-tailed and statistical significance was defined a priori at $p < 0.05$. Data analyses was performed using the statistical software package Prism v.8.0 (GraphPad Software, San Diego, CA, USA).

Results

193 **Table 1** shows participants' physical characteristics. Mild AMS was present in 2 term born and
194 4 preterm participants. However, no difference in AMS prevalence was found between groups
195 ($p = 0.656$). In both term born and preterm participants, inspired oxygen pressure was matched
196 between NNx vs. HNx vs. HNx+CO_2 (142.9 ± 0.5 vs. 139.0 ± 3.7 vs. 139.8 ± 2.5 mmHg,
197 respectively). Likewise, $P_{ET}CO_2$ was matched between NNx and HHx+clamp (38 ± 3 vs. 36 ±
198 3 mmHg, respectively).
199 **Figure 1** depicts BRS (panel A), SBP (panel B), and HR (panel C) in both groups during stages
200 1-6 of the experimental protocol. Across all conditions, BRS was lower in term born compared
201 to preterm (13.0 ± 7.5 vs. 21.2 ± 8.8 ms·mmHg^{-1}, main group effect: $p < 0.01$) participants.
202 Despite similar cardiorespiratory fitness (in terms of relative $\dot{V}O_{2peak}$), a lower BRS in HHx
203 compared to NNx was observed in term born (10.5 ± 4.9 vs. 16.0 ± 6.0 ms·mmHg^{-1},
204 respectively, $p = 0.05$), but not in preterm (27.3 ± 15.7 vs. 17.6 ± 8.3 ms·mmHg^{-1}, $p = 0.43$)
205 participants, leading to a lower BRS in HHx in term born compared to preterm (10.5 ± 4.9 vs.
206 27.3 ± 15.7 ms·mmHg^{-1}, respectively, $p < 0.01$) adults. $\dot{V}_E$, $P_{ET}CO_2$, B_f, V_T, MAP, and DBP
207 recorded during stages 1-6 of the experimental protocol are reported in **Table 2**. The ventilatory
208 responses to hypoxia, hypobaria, and hypercapnia were similarly affected by each condition (p
209 < 0.001) in the two groups ($p = 0.303$). Despite this and although we did not reach a statistical
210 significance, HCVR in NNx was higher in preterm compared to term born (1.14 ± 0.72 vs. 0.75
211 ± 0.37 L·min^{-1}·mmHg^{-1}, $p = 0.08$) participants.
212 Arterial blood gas parameters collected during each stage are listed in **Table 3**.

Discussion

213 The present study investigated BRS at sea level and high-altitude in prematurely born adults
214 and age-matched term born controls.
215 The main findings were 1) BRS was reduced by acute exposure to hypobaric hypoxia in term

born adults, leading to a significantly lower BRS at altitude compared to their preterm peers; and 2) In any hypo- or hyper-capnic conditions, no differences anymore were observed between the two groups. Altogether, our results suggest a blunted BRS to hypoxia but not to changes in capnia in preterm adults.

The hypoxia-induced decrease in BRS observed among the term born adults investigated in the present study confirmed previous findings in the literature (7, 8) and the mechanisms by which BRS is decreased have been well described. Briefly, hypoxia attenuates the parasympathetic drive to the heart which leads to a resetting of BRS to higher BP values (46). This response to acute hypoxia occurs in parallel with hypoxia-induced overactivity of the peripheral chemoreceptors which results in a coordinated response between the baro- and chemo-reflexes (45). Overall, this integrated mechanism underlies the well-known sympathoexcitation occurring during acute hypoxic exposure (46). Intriguingly, preterm adults demonstrated a blunted baroreflex response to hypoxia which suggests a dysfunction in this integrated mechanism. The higher, compared to term born controls, and unchanged, compared to normoxia, BRS found in prematurely born adults in hypoxia is a new finding that was not yet described in the literature. Although data on autonomic function in premature birth survivors beyond infancy is limited, emerging evidence suggests impaired autonomic function in adolescents (19) and young adults born preterm (22, 36). Some authors suggested reduced parasympathetic activity in adults born prematurely (22, 36). However, the present study was unable to confirm these previous speculation as we did not evaluate either sympathetic or parasympathetic HRV indices. A further explanation may find support in the adverse effects induced by postnatal hyperoxic treatment commonly applied to premature infants. As briefly outlined above, cardiac autonomic control is largely dictated by both baro- and chemo-receptors feedbacks (45), and animal models demonstrated lower carotid body density and reduced sensitivity of the afferent limb of the arterial chemoreceptor reflex in rats exposed to

241 postnatal hyperoxia (6). These anatomical and functional deficits were suggested to impair the
242 normal baroreflex growth in infants born preterm, and thus to impair autonomic responses later
243 into adulthood (19). While we did not observe lower BRS in our healthy preterm cohort
244 compared to term born peers under normoxic conditions, the blunted hypoxic chemosensitivity
245 previously reported in this cohort (3, 11) might cause or going in parallel to a blunted
246 baroreceptors response to a hypoxic stimulus. However, a parallel data collection to the
247 previous study demonstrated similar hypoxic ventilatory response in preterm adults and age-
248 matched controls born at term (29). The difference between our previous published data and
249 previous studies in the literature on hypoxic chemosensitivity in preterm population is likely
250 due to methodological differences in assessing the hypoxic ventilatory response.
251 Reduced BRS is a well-known risk factors for cardiovascular mortality and morbidity. Even
252 though we are the first to demonstrate an unchanged BRS during acute hypoxic exposure in
253 preterm adults, our results may reinforce previous speculations (10) of increased susceptibility
254 to high-altitude illnesses in this population. Emerging evidence supports specific phenotypical
255 responses to hypoxia at cardiovascular level in prematurely born adults. Barton and colleagues
256 recently demonstrated an exaggerated cardiac contractile response, especially at the level of
257 the right ventricle, in healthy preterm adults acutely exposed to severe hypoxia (FiO_2 = 12%)
258 and concluded that exposure to hypoxia may ultimately increase their risk for late right
259 ventricle heart failure (2). We also recently confirmed impaired microvascular responsiveness
260 in preterm adults, compared to term born controls, upon arrival at high-altitude (3375 m) (31),
261 thus further suggesting specific cardiovascular response to a physiological stressor, such us
262 hypoxia, in this population.
263 In the present study we also aimed to elucidate the effects of breathing different O_2 and CO_2
264 mixtures on BRS in prematurely born but otherwise healthy adults and term born controls.
265 Recent well-designed studies demonstrated that BRS is reduced during acute exposure to

hypobaric hypoxia, though this decrease was counterbalanced by breathing either supplemental O_2 (51) or additional CO_2 (8). Our findings confirm these previous results and provide further evidence that the BRS decrease occurring in acute hypoxia is partially mediated by carotid body chemoreceptors (37). Earlier studies observed a persistent increase in chemoafferent activity to the rostroventrolateral medulla via the nucleus tractus solitarius during acute hypoxia, which results in long-lasting sympathoexcitation (17, 44). Consequently, hypoxia-induced hypocapnia was shown to deactivate chemoreceptors activity leading to decreased BRS (46), while either breathing 3% hypercapnic mixture or normalizing $P_{ET}CO_2$ to sea level values (during HHx+clamp) restored BRS as in NNx likely because the sensitivity of the peripheral chemoreceptors remained unchanged. Likewise, breathing mild hyperoxic gas mixture significantly reduced their contribution and restored BRS to NNx values.

Methodological considerations

Although the present study provides novel insight into the autonomic cardiac regulation in this preterm population, there are considerations we need to acknowledge. Given that the study was conducted with males only, the present results cannot be directly extended to females. Preliminary evidence on rats suggests sex-specific difference in the preterm population (49).

Second, respiratory sinus arrythmia is a known confounding factor for the characterization of spontaneous baroreflex control at rest (43). Particularly, systolic blood pressures show a large oscillation at the respiratory frequency (20, 21), thus affecting the analyses of spontaneous cardiovagal BRS. However, as shown in **Table 2** of the present study, we did not observe any difference in ventilation or breathing frequency between our preterm and term born cohorts. Therefore, respiratory sinus arrythmia was unlikely to be a confounding factor in the present investigation.

Finally, BRS was assessed using the sequence method, which is the most common and which allows a direct interpretation of the causal link between blood pressure and HR changes.

Conclusion

This study reported a different spontaneous cardiovagal BRS response to acute hypoxia in prematurely born adults compared to term born controls. Prematurely but otherwise healthy adults showed a blunted hypoxia-induced resetting of spontaneous cardiovagal BRS. Future studies should investigate the mechanisms by which baroreflex resetting in acute hypoxia is impaired in adults born prematurely.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

G.P.M. and T.D. conceived the research and obtained the financial support. G.M., N.B., and G.P.M. contributed to the experimental design. G.M. and B.J.N. collected the data. G.M., N.B., and G.P.M. analysed and interpreted the data. G.M. drafted the manuscript. All authors critically revised the draft and approved the final version of the manuscript.

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Tables

Table 1. Participants' physical characteristics (Mean ± SD).

	Term born	Preterm	P-value
Participants' characteristics			
Gestational age (weeks)	40 ± 1	29 ± 2	***p* < 0.01**
Birth weight (g)	3621 ± 421	1132 ± 265	***p* < 0.01**
Age (years)	21 ± 2	21 ± 4	$p = 0.07$
Height (cm)	182 ± 6	178 ± 9	$p = 0.21$
Body mass (kg)	75.6 ± 6.9	72.4 ± 14.4	$p = 0.42$
BMI ($kg \cdot m^{-2}$)	22.8 ± 1.8	22.5 ± 2.7	$p = 0.74$
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	51.3 ± 7.6	48.5 ± 10.7	$p = 0.40$

Definition of abbreviations: BMI = body mass index.

Table 2. Respiratory parameters, mean arterial pressure and diastolic blood pressure recorded during the six phases of the experimental protocol in term born and prematurely born participants.

	Phase	Term born	Preterm	Group	Condition	Group × Condition
$\dot{V}_E$ ($L \cdot min^{-1}$)	NNx	11.5 ± 1.6	11.3 ± 2.0	$p = 0.30$	$\mathbf{p < 0.01}$	$p = 0.10$
	NNx+CO_2	15.0 ± 2.5	15.6 ± 3.2			
	HHx	12.9 ± 1.7	13.1 ± 1.7			
	HNx	12.6 ± 1.5	12.4 ± 1.9			
	HNx+CO_2	16.3 ± 1.7	16.2 ± 2.8			
	HHx+clamp	20.2 ± 6.4	21.3 ± 5.2			
$P_{ET}CO_2$ (mmHg)	NNx	38 ± 2	38 ± 3	$p = 0.29$	$\mathbf{p < 0.01}$	$p = 0.95$
	NNx+CO_2	43 ± 2	42 ± 3			
	HHx	32 ± 2	31 ± 1			
	HNx	33 ± 2	32 ± 2			
	HNx+CO_2	35 ± 1	35 ± 2			
	HHx+clamp	36 ± 3	36 ± 4			
B_f (BPM)	NNx	16 ± 4	15 ± 4	$p = 0.97$	$\mathbf{p < 0.01}$	$p = 0.93$
	NNx+CO_2	16 ± 3	15 ± 3			
	HHx	17 ± 3	17 ± 4			
	HNx	17 ± 3	17 ± 5			
	HNx+CO_2	18 ± 3	17 ± 4			
	HHx+clamp	18 ± 4	19 ± 3			

V_T (L)	NNx	0.7 ± 0.2	0.8 ± 0.2	$p = 0.54$	$\mathbf{p < 0.01}$	$p = 0.97$
	NNx+CO_2	1.0 ± 0.2	1.0 ± 0.3			
	HHx	0.8 ± 0.1	0.8 ± 0.2			
	HNx	0.7 ± 0.1	0.7 ± 0.2			
	HNx+CO_2	0.9 ± 0.1	1.0 ± 0.1			
	HHx+clamp	1.1 ± 0.2	1.1 ± 0.2			
MAP (mmHg)	NNx	84 ± 9	86 ± 8	$p = 0.99$	$\mathbf{p < 0.01}$	$p = 0.63$
	NNx+CO_2	85 ± 10	86 ± 10			
	HHx	94 ± 8	92 ± 8			
	HNx	94 ± 9	93 ± 9			
	HNx+CO_2	94 ± 10	94 ± 9			
	HHx+clamp	95 ± 8	97 ± 14			
DBP (mmHg)	NNx	66 ± 9	66 ± 9	$p = 0.93$	$\mathbf{p < 0.01}$	$p = 0.90$
	NNx+CO_2	66 ± 9	66 ± 10			
	HHx	74 ± 9	72 ± 9			
	HNx	73 ± 9	73 ± 9			
	HNx+CO_2	72 ± 9	74 ± 9			
	HHx+clamp	75 ± 7	75 ± 13			

Definition of abbreviations: B_f, breathing frequency; DBP, diastolic blood pressure; MAP, mean arterial pressure; NNx, normobaric normoxia; NNx+CO_2, normobaric normoxia with 3% carbon dioxide (CO_2); HHx, hypobaric hypoxia; HNx, hypobaric normoxia; HNx+CO_2, hypobaric normoxia with 3% CO_2; HHx+clamp, hypobaric hypoxia with end tidal partial pressure of CO_2 ($P_{ET}CO_2$) clamped at NNx value; $\dot{V}_E$, pulmonary ventilation, V_T, tidal volume.

Table 3. Arterial blood gas variables in term born and prematurely born participants collected during the last 30 sec of each phase of the experimental protocol.

	Phase	Term born	Preterm	Group	Condition	Group × Condition
PaO_2 (mmHg)	NNx	87 ± 9	87 ± 10	$p = 0.63$	$\mathbf{p < 0.01}$	$p = 0.80$
	NNx+CO_2	98 ± 6	101 ± 6			
	HHx	53 ± 2	53 ± 3			
	HNx	87 ± 5	88 ± 6			
	HNx+CO_2	99 ± 3	100 ± 4			
	HHx+clamp	66.3 ± 3.9	68.1 ± 1.8			
$PaCO_2$ (mmHg)	NNx	42.4 ± 2.1	40.9 ± 3.3	$p = 0.26$	$\mathbf{p < 0.01}$	$p = 0.23$
	NNx+CO_2	44.2 ± 1.7	43.6 ± 3.3			
	HHx	34.4 ± 1.3	33.6 ± 2.9			
	HNx	36.5 ± 1.2	35.5 ± 2.5			
	HNx+CO_2	37.6 ± 1.1	36.9 ± 2.3			
	HHx+clamp	37.4 ± 2.7	39.2 ± 3.3			
pH	NNx	7.41 ± 0.01	7.41 ± 0.02	$p = 0.20$	$\mathbf{p < 0.01}$	$p = 0.32$
	NNx+CO_2	7.40 ± 0.02	7.39 ± 0.02			
	HHx	7.44 ± 0.02	7.44 ± 0.01			
	HNx	7.43 ± 0.02	7.42 ± 0.02			
	HNx+CO_2	7.42 ± 0.02	7.41 ± 0.02			
	HHx+clamp	7.42 ± 0.02	7.40 ± 0.00			

$[H^+]$ (nM)	NNx	38.6 ± 1.3	38.8 ± 2.0	$p = 0.13$	$\mathbf{p < 0.01}$	$p = 0.15$
	NNx+CO_2	39.7 ± 1.8	40.8 ± 2.3			
	HHx	36.0 ± 1.4	36.0 ± 1.2			
	HNx	37.4 ± 1.6	37.8 ± 1.6			
	HNx+CO_2	38.3 ± 1.6	38.6 ± 1.4			
	HHx+clamp	37.9 ± 2.2	40.1 ± 0.5			
$[HCO_3^-]$ ($mEq \cdot L^{-1}$)	NNx	26.7 ± 1.8	25.7 ± 2.5	$p = 0.10$	$\mathbf{p < 0.01}$	$p = 0.90$
	NNx+CO_2	27.2 ± 1.5	26.1 ± 2.4			
	HHx	23.6 ± 1.1	23.0 ± 1.7			
	HNx	24.1 ± 1.2	23.1 ± 1.5			
	HNx+CO_2	24.2 ± 1.1	23.6 ± 1.5			
	HHx+clamp	24.4 ± 1.1	24.1 ± 1.7			
SaO_2 (%)	NNx	96.6 ± 1.1	96.5 ± 1.8	$p = 0.62$	$\mathbf{p < 0.01}$	$p = 0.97$
	NNx+CO_2	98.0 ± 0.4	98.2 ± 0.4			
	HHx	88.6 ± 1.7	89.0 ± 1.8			
	HNx	97.2 ± 0.9	97.4 ± 0.5			
	HNx+CO_2	98.1 ± 0.4	98.2 ± 0.4			
	HHx+clamp	93.3 ± 1.1	93.9 ± 1.3			

Definition of abbreviations: $[H^+]$, hydrogen concentration; $[HCO_3^-]$, bicarbonate concentration; NNx, normobaric normoxia; NNx+CO_2, normobaric normoxia with 3% carbon dioxide (CO_2); HHx, hypobaric hypoxia; HNx, hypobaric normoxia; HNx+CO_2, hypobaric normoxia with 3% CO_2; HHx+clamp, hypobaric hypoxia with end tidal partial pressure of CO_2 ($P_{ET}CO_2$) clamped

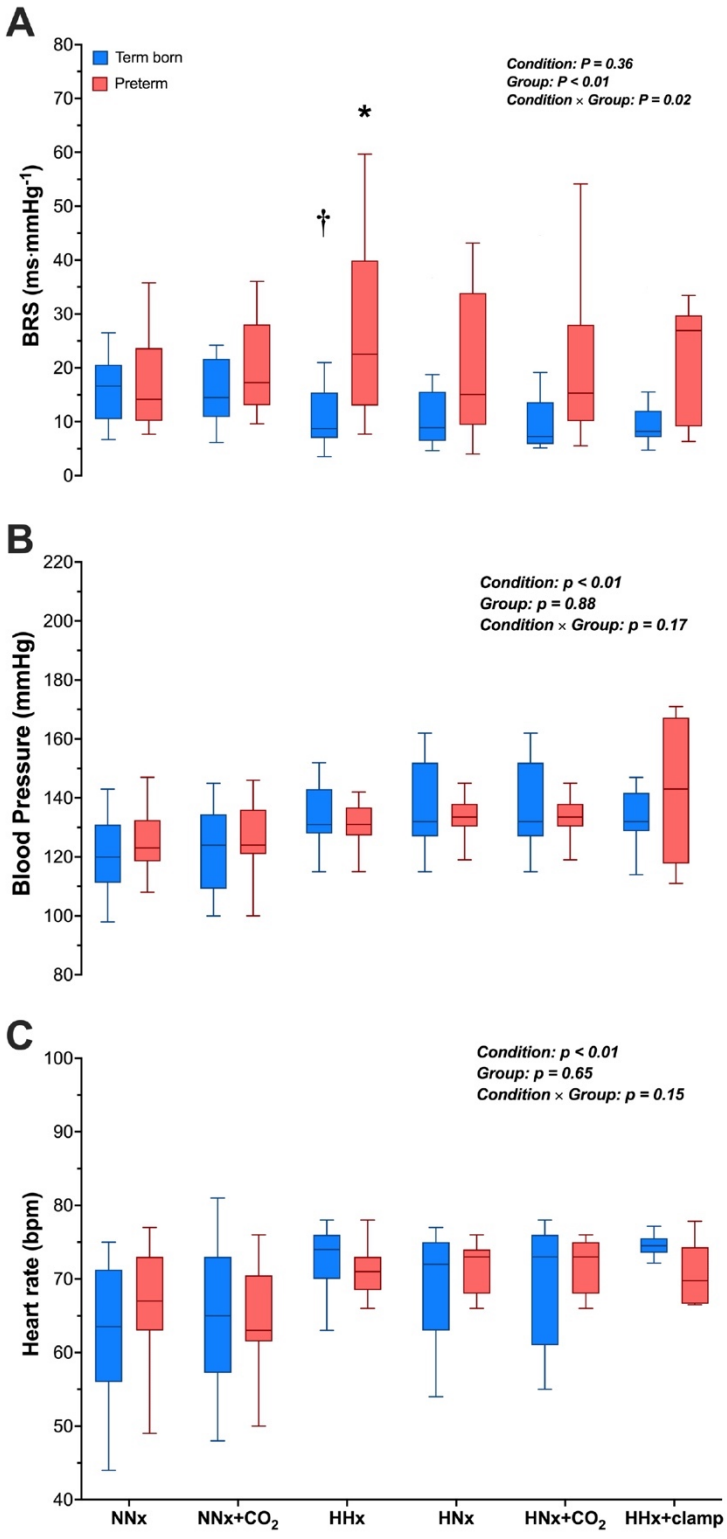
475 at NNx value; $PaCO_2$, arterial partial pressure of CO_2; PaO_2, arterial partial pressure of oxygen

476 (O_2); SaO_2, arterial O_2 saturation.

Figure Captions

Figure 1. Tukey boxplots, horizontal line inside boxes: median; upper and lower lines of boxes: 75^{th} and 25^{th} percentiles, respectively; upper and lower whiskers: highest and lowest data points within the 1.5 inter quartile range. Baroreflex sensitivity (BRS; Panel A), systolic blood pressure (SBP; Panel B), and heart rate (HR; Panel C) in term born (in blue) and prematurely born (in red) adults during the 6 stages of the experimental protocol. NNx, normobaric normoxia; NNx+CO_2, normobaric normoxia with 3% CO_2; HHx, hypobaric hypoxia (3375 m); HNx, hypobaric normoxia; HNx+CO_2, hypobaric normoxia with 3% CO_2; HHx+clamp, hypobaric hypoxia with $P_{ET}CO_2$ clamped at NNx value. *Significantly different from term born; †significantly different from NNx.

Figure 1


A
Term born
Preterm
Condition: P = 0.36
Group: P < 0.01
Condition × Group: P = 0.02
BRS (ms·mmHg⁻¹)
*
†
B
Condition: p < 0.01
Group: p = 0.88
Condition × Group: p = 0.17
Blood Pressure (mmHg)
C
Condition: p < 0.01
Group: p = 0.65
Condition × Group: p = 0.15
Heart rate (bpm)
NNx
NNx+CO2
HHx
HNx
HNx+CO2
HHx+clamp

486

12. Article 4

Impaired cerebrovascular CO_2 reactivity at high-altitude in prematurely born adults

Article 4 – Impaired cerebrovascular CO_2 reactivity at high-altitude in prematurely born adults

Manferdelli G, Narang BJ, Bourdillon N, Giardini G, Debevec T & Millet GP. *J Physiol.* – in revision.

Impaired cerebrovascular CO_2 reactivity at high-altitude in prematurely born adults

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Key points summary:

- Cerebral hemodynamics and cerebrovascular reactivity in normoxia are known to be similar between term born and prematurely born adults.
- In contrast, acute high-altitude exposure unveiled different cerebrovascular responses to hypoxia, hypercapnia, and hypocapnia. In particular, cerebral vasodilation was impaired in prematurely born adults, leading to an exaggerated cerebral vasoconstriction.
- Cardiovascular and ventilatory responses to both hypo- and hyper-capnia, at sea level and high-altitude, were similar between controls and prematurely born adults. Other mechanisms may therefore underlie the observed blunted cerebral vasodilatory responses in preterm adults at high-altitude.

Abstract

Premature birth impairs cardiac and ventilatory responses to both hypoxia and hypercapnia, but little is known about cerebrovascular responses. Both at sea level and after 2 days at high-altitude (3375 m), sixteen young preterm (gestational age, 29 ± 1 weeks), and fifteen age-matched term born (40 ± 0 weeks) adults were exposed to two consecutive 4-min hyperoxic hypercapnic conditions (3%CO_2-97%O_2; 6%CO_2-94%O_2), followed by two periods of voluntary hyperventilation-induced hypocapnia. We measured middle cerebral artery blood velocity (MCA*v*), end-tidal CO_2 ($P_{ET}CO_2$), pulmonary ventilation ($\dot{V}_E$), beat-by-beat mean arterial pressure (MAP), and arterialized capillary blood gases. Baseline MCA*v* increased at high-altitude compared to sea level in term born (+24 ± 39%, $P = 0.036$), but not in preterm (-4 ± 27%, $P = 0.278$), adults. $P_{ET}CO_2$, $\dot{V}_E$, and MAP were similar between groups at sea level and high-altitude. Hypocapnic cerebrovascular reactivity was higher at high-altitude compared to sea level in term born (+173 ± 326%, $P = 0.026$), but not in preterm (-21 ± 107%, $P = 0.572$) adults. Hypercapnic reactivity was altered at altitude only in preterm adults (+125 ± 144%, $P < 0.001$). Collectively, at high-altitude, term born participants showed higher hypocapnic ($P = 0.012$) and lower hypercapnic ($P = 0.020$) CO_2 reactivity compared to their preterm peers. In conclusion, high-altitude exposure revealed different cerebrovascular responses in preterm, compared to term born adults, despite similar ventilatory responses. These findings suggest a blunted cerebrovascular response at high-altitude in preterm adults, which may predispose these individuals to an increased risk of high-altitude illnesses.

1. Introduction

Preterm birth (<37 weeks gestation) affects over 10% of live births worldwide (Blencowe *et al.*, 2013) and those born extremely preterm (≤32 weeks gestation) are considered to be at higher risk for both short- and long-term morbidity (Raju *et al.*, 2017). The long-term sequelae of an early delivery on pulmonary (Bates *et al.*, 2014; Duke *et al.*, 2022; Narang *et al.*, 2022) and cardiovascular functions (Engan *et al.*, 2021; Manferdelli *et al.*, 2022; Schuermans & Lewandowski, 2022; Manferdelli *et al.*, 2023) are well described, but the effects on the cerebrovascular system require further investigation.

Cerebrovascular reactivity to carbon dioxide (CO_2) is often used to assess cerebrovascular endothelial health (Portegies *et al.*, 2014), as it integrates several mechanisms within the cerebral vasculature in response to changes in arterial partial pressure of CO_2 (P_aCO_2) (Ainslie & Duffin, 2009). This cerebrovascular response might be compromised in prematurely born newborns, as a consequence of incomplete development of the cerebral circulation, including impaired ongoing angiogenesis and vasoregulatory immaturity (Brew *et al.*, 2014). However, cerebrovascular reactivity to CO_2 was reported to be either normal (Greisen & Trojaborg, 1987; Pryds *et al.*, 1990; Mosca *et al.*, 1999; Jayasinghe *et al.*, 2003) or increased (Aly *et al.*, 2019) in preterm newborns shortly after birth. Of note, these studies performed indirect measurements (i.e., brain NIRS) to assess cerebral oxygenation changes in response to spontaneous end-tidal CO_2 ($P_{ET}CO_2$) fluctuations. Surprisingly, data on cerebrovascular regulation to changes in arterial blood gases (P_aCO_2 and arterial O_2 partial pressure, P_aO_2) in adults born prematurely are still missing.

The fundamental mechanisms underlying cerebrovascular CO_2 reactivity and their complex interaction with ventilatory sensitivity to changes in P_aCO_2 and $P_{ET}CO_2$ are comprehensively discussed elsewhere (Ainslie & Duffin, 2009; Carr *et al.*, 2021a). Briefly, as the cerebral vasculature embeds the neural centres controlling respiration, a proper regulation of cerebral blood flow is fundamental to ensure an adequate balance of P_aCO_2 and hydrogen ions which both directly impact ventilatory sensitivity (Carr *et al.*, 2021a). Therefore, the sensitivity of cerebral blood flow regulation directly impacts the ventilatory response to changes in arterial blood gas balance.

From one perspective, altitude significantly modulates cerebral blood flow via hypoxia-induced vasodilation within a few minutes of exposure, to maintain cerebral oxygen delivery (Ainslie *et al.*, 2007; Lucas *et al.*, 2011; Willie *et al.*, 2014). On the other hand, due to the hypoxic ventilatory response (HVR), the hyperventilation-induced hypocapnia represents a strong vasoconstrictor stimulus (Willie *et al.*, 2014), which briefly overrides hypoxic cerebral

vasodilation (Steinback & Poulin, 2008). A balance between the competing effects of hypoxia and hypocapnia on cerebrovascular and ventilatory control is established within hours of exposure to altitude (Carr *et al.*, 2021a). However, the effects of prolonged high-altitude exposure on cerebrovascular CO_2 reactivity are still unclear and contradictory, with increased (Ainslie *et al.*, 2012; Fan *et al.*, 2015; Fluck *et al.*, 2015; Aebi *et al.*, 2020), decreased (Ainslie *et al.*, 2007; Rupp *et al.*, 2014), unchanged (Willie *et al.*, 2015), or selectively altered (Lucas *et al.*, 2011) cerebrovascular reactivity to changes in P_aCO_2 having been reported.

The main aim of the present study was to investigate the cerebrovascular responses to CO_2 at high-altitude in prematurely born but otherwise healthy adults, compared to their term born age-matched peers. Moreover, to gain further insight, ventilatory and cardiovascular responses were also investigated. Based on previous evidence of prematurity-induced vasoregulatory immaturity (Brew *et al.*, 2014), we hypothesized that prematurely born adults will display blunted cerebrovascular CO_2 reactivity under normoxia, and furthermore that this phenomenon will be further exacerbated at high-altitude.

Methods

Participants

Thirty-one young healthy men volunteered and gave written informed consent to participate in this study. Sixteen participants were born preterm and fifteen were born at term. The preterm born participants were recruited via the National preterm birth register managed by the University Clinical Centre in Ljubljana, Slovenia using medical record screening and telephone/email-based individual interviews. The inclusion criteria for the preterm group were gestational age ≤32 weeks, birth weight ≤1500 g, O_2 therapy at birth, and absence of diagnosed bronchopulmonary dysplasia. The inclusion criteria for the term born participants were gestational age ≥38 weeks and birth weight ≥2500 g. Birth-related inclusion criteria for all participants were checked and confirmed during the initial birth/medical record screening procedure conducted during recruitment. Exclusion criteria for all participants included permanent altitude residence (≥1000 m), cardiopulmonary, haematological and/or kidney disorders, chronic medication use, smoking and altitude/hypoxia exposure (≥2000 m) within the last month prior to the study. Participants were matched for age, height, and body mass. This study was performed according to the Declaration of Helsinki. The experimental protocol was pre-registered at ClinicalTrials.gov (NCT04739904), and ethical approvals were

obtained from both the University of Ljubljana, Faculty of Sport ethics committee (8/2020-316) and the Aosta Hospital Ethical Committee (06/05/2021.0038781.I).

Experimental design and ascent protocol

Each participant underwent two experimental trials, one near sea level (Ljubljana, Slovenia; barometric pressure ~737±2 mmHg) and the other at high-altitude (3375m; Torino hut, Aosta Valley, Italy, on the Mont Blanc massif; barometric pressure ~503±3 mmHg). Participants travelled from Ljubljana to Courmayeur (1300 m) by car, then travelled by cable car to reach Torino hut in approximately 15-20 minutes. In order to avoid any confounding influence of acute mountain sickness (AMS), experimental sessions at altitude were carried out on the second day after arrival at Torino hut.

Experimental protocol

All experiments were performed at the same time of day (between 9:00 and 12:00) both at sea level and at altitude. Before each trial, participants were instructed to abstain from exercise for 12 hours and avoid alcohol and caffeine for 24 hours. They also consumed no heavy meals within 4 hours of testing. All experiments were conducted with the participant comfortably seated on a chair. Each testing session began with equipment instrumentation followed by 5 min of seated rest. A 4 min baseline data collection period then began, during which participants were breathing ambient air, before 8 min of incremental hyperoxic hypercapnia, and then 8 min of voluntary hyperventilation to induce steady-state hypocapnia. Incremental hyperoxic hypercapnia was induced by switching the inspired gas from ambient air to 3% CO_2 (in 97% O_2) for 4 min, then to 6% CO_2 (in 94% O_2) for a further 4 min. The $P_{ET}CO_2$ values during the last 10 s of each hyperoxic hypercapnic exposure were recorded. Following the incremental hyperoxic hypercapnic exposures, participants breathed ambient air to ensure that ventilatory and cerebrovascular parameters to baseline values. Once a baseline steady-state had been achieved, participants were instructed to increase their ventilation (both frequency and depth) in such a way as to induce two levels (4 min each) of steady-state hypocapnia to match, in an equal and opposite direction, the rise in $P_{ET}CO_2$ that had occurred during the two hyperoxic hypercapnic conditions. As recently demonstrated, 4 min is a sufficient period to ensure a physiological steady state in both $P_{ET}CO_2$ and MCA*v* (Carr *et al.*, 2021b). In the present study, hyperoxic hypercapnia was intentionally used to eliminate the influence of peripheral chemoreceptor activation induced by hypoxic exposure, and to remove the consequent effects on cerebrovascular tone. The hypercapnic stages were

conducted at the start of the test to allow the individualised changes in $P_{ET}CO_2$ induced by progressive hypercapnia to be recorded, and thus calculate the target $P_{ET}CO_2$ values to be reached during the hypocapnic stages. This order was also important since hypocapnia, but not hypercapnia, might cause persistent vasoconstriction in the cerebral arteries, potentially altering the normal MCA*v* response to increasing arterial CO_2 concentration (Ide *et al.*, 2003). Despite this, a full recovery of all ventilatory and cerebrovascular parameters to baseline was ensured between hypercapnia and hypocapnia.

Measurements

Respiratory variables. Participants breathed through a leak-free respiratory mask (Hans-Rudolph 7450 series, Kansas City, MO, USA; dead space: 75 mL) attached to a T-shaped two-way non-rebreathing valve (Hans Rudolph, Kansas City, MO, USA). $P_{ET}CO_2$ and pulmonary ventilation ($\dot{V}_E$) were measured using a breath-by-breath system (Ergocard Professional, Medisoft, Sorinnes, Belgium).

Cerebrovascular and cardiovascular variables. Blood flow velocity in both the right and left middle cerebral artery was measured using a 2-MHz pulsed Doppler ultrasound system (ST3, Spencer technology, Seattle, WA, USA). The right and left Doppler probes were positioned over the right and left temporal windows, respectively, and held in place with an adjustable plastic headband (Marc 600 Headframe, Spencer technology, Seattle, WA, USA). The right and left MCA*v* were insonated through the trans-temporal windows following research technique and guidelines described in detail elsewhere (Willie *et al.*, 2011). Beat-by-beat MAP and heart rate were monitored non-invasively using a finger photoplethysmography device (NIBP100D, Biopac Systems Inc., Goleta, CA, USA) attached to a double cuff placed on the index and middle fingers of the left hand. To ensure accurate measurements of MAP, the device was calibrated using a direct measurement of left arm blood pressure by a digital blood pressure device. Due to technical issues at sea level, MAP was monitored only in 9 term born and 14 preterm participants.

Arterialized capillary blood gas variables. For each participant, capillary blood samples were taken from the earlobe during the last 30 seconds of each 4-min stage (including baseline). Arterialization of capillary blood was achieved by applying a vasodilation cream (Capsolin, SIT s.r.l., Mede, Italy). Arterial blood variables (P_aO_2, P_aCO_2, pH, hydrogen ion concentration ($[H^+]$), bicarbonate concentration ($[HCO_3^-]$), base excess, arterial oxygen saturation (S_aO_2), and total haemoglobin concentration ([Hb])) were immediately analysed

using an arterial blood gas analyser (ABL-90 FLEX, Radiometer, Copenhagen, Denmark).

Acute Mountain Sickness. Symptoms of AMS were evaluated on the morning of the test using the Lake Louise Scale (Roach *et al.*, 2018). Accordingly, AMS was diagnosed when headache was present together with at least one additional symptom, and the total score was three or higher.

Data Acquisition

Cerebrovascular hemodynamics and cardiovascular data were measured continuously at 200 Hz and 1000 Hz, respectively, using an analog-to-digital converter (Powerlab 16/30, ADInstruments, Dunedin, New Zeland, and MP150, Biopac Systems Inc, Goleta, CA, USA, respectively). These devices were interfaced to a computer using dedicated software (Labchart v. 8.1.17, ADInstruments, Dunedin, New Zealand, and Acknowledge v. 4.2, Biopac Systems Inc, Goleta, CA, USA, respectively), and data were stored for later analyses.

Data Analyses

The hypercapnic ventilatory response to 6%CO_2 ($HCVR_6$) was calculated as the ratio between the differences in $\dot{V}_E$ and $P_{ET}CO_2$ from ambient air to hypercapnia, to account for potential differences in P_aCO_2 that likely impact both central and peripheral chemoreceptor responses.

MCA*v* and $P_{ET}CO_2$ data were first reduced to 10-s averages across the entire testing period and time-aligned. The MCA*v* response to $P_{ET}CO_2$ was then fitted with a sigmoid curve of type:

$$\text{MCA}v = min + (A \,/\, (1 + e^{-(P_{ET}CO_2 - X_0)/range}))$$

where, *min* represents the minimum MCA*v* reached during the hypocapnic (hyperventilation) phase, *A* is the amplitude of the response, X_0 is the $P_{ET}CO_2$ value at the center of the sigmoid curve, and range is the $P_{ET}CO_2$ range of the linear portion of the sigmoid.

Hypoxic cerebrovascular reactivity was calculated to assess the effect of hypobaric hypoxia on changes in resting MCA*v* from sea level values using three different equations (Lucas *et al.*, 2011) - the ratio between the percent change in MCA*v* and 1) the change in S_aO_2 (ΔMCA*v*/ΔS_aO_2); 2) the change in P_aCO_2 (ΔMCA*v*/ΔP_aCO_2); and 3) the change in P_aO_2/P_aCO_2 (ΔMCA*v*/$\Delta(P_aO_2/ P_aCO_2)$) ratio.

Hypercapnic and hypocapnic cerebrovascular reactivity values were determined as the

percent change in MCA*v* from baseline to hypercapnia and hypocapnia, respectively, per unit change in $P_{ET}CO_2$.

Indices of cerebrovascular resistance (CVR*i*) and cerebrovascular conductance (CVC*i*) were calculated for each stage as:

$$CVRi = \frac{MAP}{MCAv_{mean}} \qquad CVCi = \frac{MCAv_{mean}}{MAP}$$

Blood pressure and MCA*v* during the last 30 cardiac cycles of each stage were also extracted, time-aligned, and used to estimate the critical closing pressure (CrCP). CrCP was estimated as the blood pressure value corresponding to the x-axis intercept of the linear regression between arterial blood pressures during the last 30 cardiac cycles plotted against the corresponding MCA*v* values.

Statistical analysis

All data are presented as mean ± SD throughout the manuscript. A three-way (group × condition × altitude) ANOVA was performed to compare the changes in respiratory, cardiovascular, and arterial blood gas parameters during all the tested conditions both at sea level and at high-altitude between preterm and term born adults. A two-way (group × altitude) repeated measures ANOVA was performed to compare the changes in cerebrovascular parameters and cerebrovascular reactivity to CO_2 at sea level and at high-altitude between preterm and term born adults. Significant interaction effects were analysed by Sidak correction. All *p*-values are two-tailed and statistical significance was defined a priori at $p<0.05$. Data analyses was performed using the statistical software package Prism v.6.0 (GraphPad Software, San Diego, CA, USA).

Results

Participants' physical characteristics are reported in **Table 1**.

Participants' spirometry, lung diffusion capacity to CO_2, and hypoxic ventilatory response were presented elsewhere (Manferdelli *et al.*, 2022). No difference was observed between the two groups in pulmonary function and ventilatory response to hypoxia.

On the morning of the high-altitude test, one term born and one preterm participant reported mild AMS symptoms (score = 3).

Respiratory variables

Figure 1 shows $\dot{V}_E$ and $P_{ET}CO_2$ during each condition (ambient air, hypercapnia, and hypocapnia) at sea level and at high-altitude in term born and preterm participants. Compared to sea level, exposure to altitude induced an increase in $\dot{V}_E$ (11.2 ± 1.9 to 13.2 ± 1.6 L·min^{-1}, main altitude effect: P<0.001) and a concomitant fall in $P_{ET}CO_2$ (37 ± 6 to 30 ± 2 mmHg, main altitude effect: P<0.001), which were similar between groups (P = 0.904 and P = 0.137, respectively). The $HCVR_6$ was similar between groups both at sea level (term born: 1.2 ± 0.3 L·min^{-1} mmHg^{-1}; preterm: 1.3 ± 0.9 L·min^{-1} mmHg^{-1}, P = 0.950) and high-altitude (term born: 2.4 ± 2.7 L·min^{-1} mmHg^{-1}; preterm: 1.7 ± 1.3 L·min^{-1} mmHg^{-1}, P = 0.427), though $HCVR_6$ was increased by altitude in both term born and preterm participants (+91 ± 179% and +37 ± 61%, respectively; main altitude effect: P = 0.029).

Cerebrovascular and cardiovascular variables

Figure 2 shows the MCA*v* response to changes in $P_{ET}CO_2$ both at sea level (Panel A and C) and at high-altitude (Panel B and D) in a typical term born and preterm participant. Of note, MCA*v* while breathing ambient air was similar between term born and preterm participants at sea level (48.5 ± 9.9 vs. 53.3 ± 9.8 cm·s^{-1}, respectively, P = 0.295; **Figure 3**). However, altitude exposure induced an increase in MCA*v* in term born (+24 ± 39%, P = 0.035) but not in preterm (-4 ± 27%, P = 0.295) participants. In turn, at altitude, MCA*v* while breathing ambient air (i.e., hypobaric hypoxia) was significantly higher in term born compared to preterm adults (P = 0.046; **Figure 3**). Hypercapnic cerebrovascular reactivity was similar between term born and preterm participants at sea level (4.7 ± 1.8 vs. 3.5 ± 2.1 %·mmHg^{-1}, respectively, P = 0.461; **Figure 4**), while, at high-altitude, it was significantly lower in term born compared to preterm participants (4.4 ± 2.0 vs. 7.5 ± 4.7 %·mmHg^{-1}, respectively, P = 0.012; **Figure 4**). This was secondary to an increased cerebrovascular reactivity to hypercapnia at altitude compared to sea level in the preterm group (+125 ± 144%, P < 0.001; **Figure 4**). In contrast, hypocapnic cerebrovascular reactivity at high-altitude was significantly higher in term born compared to preterm participants (3.9 ± 2.2 vs. 2.1 ± 2.6 %·mmHg^{-1}, respectively, P = 0.012; **Figure 4**), due to an increase cerebrovascular reactivity to hypocapnia at high-altitude compared to sea level in the former group (+173 ± 326%, P = 0.026; **Figure 4**). The amplitude of the MCA*v* response was similar between groups (term born: 109.9 ± 19.2 cm·s^{-1}; preterm: 109.4 ± 20.0 cm·s^{-1}, P = 0.948) and altitudes (sea level: 109.2 ± 21.8 cm·s^{-1}; high-altitude: 110.1 ± 16.9 cm·s^{-1}, P = 0.832). Cerebral vasodilation and vasoconstriction reserves are shown in **Figure 5**. X_0, represents the optimal operating point of the vessels' capacity to dilate and constrict, was significantly higher at sea

level compared to high-altitude (40 ± 3 vs. 30 ± 2 mmHg, respectively, $P < 0.001$) and in term born adults compared to preterm participants (36 ± 6 vs. 35 ± 5 mmHg, respectively, $P = 0.025$). Hypoxic cerebrovascular reactivity, calculated using three different methods, is reported in **Table 2**. CVR*i*, CVC*i*, and the main cardiovascular parameters are reported in **Table 3**.

Arterialized capillary blood gas variables

Arterial blood gas variables are reported in **Table 4.** The P_aO_2-to-P_aCO_2 ratio was significantly lower at high-altitude compared to sea level (1.6 ± 0.1 vs. 2.2 ± 0.3, respectively, $P < 0.001$), with no difference between term born and preterm participants. In term born adults, the altitude-induced percent increase in MCA*v* was significantly correlated with the percent change in arterial blood gas balance ($\Delta PaO_2/PaCO_2$; $r^2 = 0.450$, $P = 0.035$).

Discussion

We investigated the cerebrovascular, ventilatory and cardiovascular responses to CO_2 at sea level and at high-altitude in healthy preterm born adults and their age-matched counterparts born at term. The general aim was to elucidate the long-term sequelae of premature birth on cerebrovascular reactivity to CO_2 both at sea level and after two days of high-altitude exposure. To the best of our knowledge, this paper is the first to investigate cerebral hemodynamics and cerebrovascular reactivity in prematurely born adults, both at sea level and at high-altitude.

The main findings were a) similar cerebrovascular reactivity to CO_2 at sea level in term born and preterm adults; and b) different cerebrovascular responses to hypoxia, hypercapnia and hypocapnia in preterm compared to term born adults at high-altitude. In contrast with our initial hypothesis, cerebral hemodynamics and cerebrovascular reactivity to CO_2 at sea level, but not at high-altitude, were comparable between controls and preterm adults. These results provide further support to previous findings from our research group (Debevec *et al.*, 2019; Manferdelli *et al.*, 2022) and others (Farrell *et al.*, 2015; Barton *et al.*, 2021) on a specific phenotypical response to hypoxia in the preterm population.

A growing body of research demonstrates persistent long-term effects of premature birth on the cardiovascular system, leading to an increased risk of cardiovascular events (Crump *et al.*, 2019; Lewandowski *et al.*, 2020) and stroke (Crump *et al.*, 2021). However, the extent to which premature birth affects cerebrovascular health and function remained to be clarified. Cerebrovascular endothelial health is typically assessed in terms of changes in cerebral blood

flow velocity in response to changes in P_aCO_2 (Portegies *et al.*, 2014). Despite significantly immature respiratory system development and respiratory control (Erickson *et al.*, 2021), cerebral blood flow regulation and the pressor response - within physiological variations of P_aCO_2 - seem to be maintained in preterm newborns shortly after birth (Pryds *et al.*, 1990). Likewise, CO_2 cerebrovascular reactivity was reported to be normal in infants supported with mechanical ventilation (Greisen & Trojaborg, 1987; Pryds *et al.*, 1990). However, it was still unclear whether these results can apply to adults born prematurely. Our findings showed that cerebrovascular reactivity to CO_2 and cerebral hemodynamics at sea level were comparable between healthy preterm adults and their term born peers. In line with this, both the ventilatory and the cardiovascular responses to changes in P_aCO_2 were similar between the groups, suggesting the mechanisms regulating the physiological responses to hyper- and hypocapnia at rest are preserved in adults born prematurely. Interestingly, a recent work from our research group showed an exaggerated ventilatory response to normoxic hypercapnia (6% CO_2) in adults born preterm compared to term controls (Manferdelli *et al.*, 2021). However, in the present study, $HCVR_6$ was similar between preterm and term born adults. The discrepancy with our previous results may be due to the different O_2 concentrations in the hypercapnic gas mixture, suggesting that hyperoxia may have a role in alleviating the differences in the ventilatory response to hypercapnia between the two populations.

Acute exposure to high-altitude is known to evoke several physiological responses, including increases in cerebral blood flow and $\dot{V}_E$, to maintain oxygen delivery to the brain and the active tissues (Severinghaus *et al.*, 1966; West, 1989; Willie *et al.*, 2014). The magnitude of the increase in cerebral blood flow under hypoxic conditions is the result of changes in arterial blood gas balance (Lucas *et al.*, 2011) and ventilatory sensitivity to both hypoxia and hypocapnia (Carr *et al.*, 2021a). In turn, these mechanisms were shown to affect cerebrovascular reactivity to CO_2, with reduced hypercapnic cerebrovascular reactivity and increased hypocapnic cerebrovascular reactivity during the first days at high-altitude compared to sea level (Lucas *et al.*, 2011).

Previous studies investigating the physiological responses to acute normobaric or hypobaric hypoxia in prematurely born adults suggest specific cardiovascular and ventilatory responses to hypoxia in this population. Barton and colleagues recently observed an exaggerated resting cardiac contractile response to acute hypoxia, especially in right ventricular function, in preterm compared to term born participants (Barton *et al.*, 2021). However, the present study did not observe different cardiovascular or pressor responses between preterm and term born participants at sea level or at high-altitude. At the pulmonary level, previous studies have

produced contrasting results, with either lower (Bates *et al.*, 2014; Debevec *et al.*, 2019) or normal (Manferdelli *et al.*, 2022) hypoxic ventilatory responses in preterm compared to term born adults. In the present study, the ventilatory responses to both hypoxia and hypercapnia were similar between preterm and term born participants. However, despite similar ventilatory responses, MCA*v* (taken as an index of cerebral blood flow) increased at high-altitude compared to sea level in term born, but not in preterm born adults, suggesting a blunted cerebral vasodilatory response to hypoxia in the preterm cohort. Similar results were also recently found in an animal model simulating premature birth in lambs, though in this study the decreased MCA*v* in response to hypoxia was due to a drop in the pressor response (Agrawal *et al.*, 2020). In contrast, the magnitude of the MCA*v* increase in response to altitude observed in the present study among control participants (+24 ± 39%) is comparable with previous works conducted at similar altitudes (+26 ± 22% at 3475 m (Jensen *et al.*, 1990) and +23 ± 12% at 3810 m (Severinghaus *et al.*, 1966)) and, as previously demonstrated (Lucas *et al.*, 2011), related to changes in arterial blood gas balance. Moreover, the higher hypercapnic cerebrovascular reactivity and lower hypocapnic cerebrovascular reactivity at altitude in preterm participants compared to their term born peers further suggest that the mechanisms underlying cerebral vasodilation may not respond to acute exposure to hypoxia. The decreased hypercapnic and increased hypocapnic cerebrovascular reactivity at high-altitude were attributed to a resetting of the total cerebral vasomotor reactivity (Jansen *et al.*, 1999), defined as the sum of the vasodilation reserve while breathing 6%CO_2 and the vasoconstriction reserve during voluntary hyperventilation (Ringelstein *et al.*, 1988). A recent mechanistic study demonstrated a key role of haemoglobin-based nitric oxide signalling in driving cerebral hypoxic vasodilation (Hoiland *et al.*, 2023). This latter mechanism may be particularly critical in prematurely born adults, as neonatal exposure to high levels of oxygen can lead to impaired endothelium-mediated vasodilation (Yzydorczyk *et al.*, 2008), secondary to endothelial nitric oxide synthase uncoupling and decreased nitric oxide production in adult rats (Yzydorczyk *et al.*, 2013). Overall, the impaired cerebral vasodilatory response to hypoxia in preterm adults might predispose these individuals to an increased risk of different high-altitude related sicknesses, and, given the growing number of individuals reaching high-altitude every year, undoubtedly warrants further investigation.

Methodological considerations

Even though the present study was the first to assess cerebrovascular hemodynamics and cerebrovascular CO_2 reactivity in prematurely born adults at both sea level and high-altitude,

a few potential limitations should be considered in the interpretation of these results. First, changes in MCA*v* are proportional to changes in cerebral blood flow only if middle cerebral artery diameter remains unchanged. Although we cannot completely exclude that our findings may have been influenced by changes in vessel diameter, direct measurement of middle cerebral artery diameter in humans under a variety of experimental conditions and to various stimuli have demonstrated that the diameter remains relatively constant (Giller *et al.*, 1993). Therefore, we are confident that our findings on the MCA*v* response to hypoxia and hyper- and hypocapnia likely reflect changes in cerebral blood flow. Moreover, the present study investigated only male survivors of preterm birth, despite preliminary evidence in rodents that sex differences may exist in the physiological response to different gas mixtures in preterm birth survivors (Tetri *et al.*, 2018). Therefore, we stress the need for further research on sex-specific responses to either normoxia or hypoxia in preterm birth adult survivors.

Conclusions

Overall, although cerebral hemodynamics and cerebrovascular CO_2 reactivity seems well-maintained in prematurely born adults at sea level, the exposure to high-altitude revealed different cerebrovascular responses to hypoxia, hypercapnia, and hypocapnia. We observed a blunted hypoxia-induced cerebral vasodilation in preterm participants, possibly due to impaired endothelium-mediated mechanisms rather than alterations in cardiovascular and/or respiratory responses to hypoxia. The present findings indicate the potential for impaired acute and altitude acclimatization responses in adults born prematurely, which could ultimately lead to an increased risk of high-altitude illnesses. Future studies with particular focus on the molecular and endothelium-related mechanisms underlying this blunted cerebrovascular response to hypoxia in this population are warranted.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

GPM and TD conceived the research and obtained the financial support. GM, NB, GG, and GPM contributed to the experimental design. GM and BJN collected the data. GM, NB, and GPM analysed and interpreted the data. GM drafted the manuscript. All authors critically revised the draft and approved the final version of the manuscript.

Competing Interests

None to declare.

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Tables

Table 1. Participants' baseline characteristics (Mean ± SD).

	Term born	Preterm	P-value
Gestational age (weeks)	40 ± 1	29 ± 2	P < 0.001
Birth weight (g)	3615 ± 442	1142 ± 270	P < 0.001
Age (years)	21 ± 2	21 ± 4	P = 0.625
Height (cm)	182 ± 7	177 ± 8	P = 0.121
Body mass (kg)	75.1 ± 7.3	70.3 ± 11.9	P = 0.183
Body mass index ($kg \cdot m^{-2}$)	22.8 ± 2.0	22.2 ± 2.5	P = 0.505

620 **Table 2.** Resting hypoxic cerebrovascular reactivity at high-altitude (3375 m) in term born and preterm born participants calculated in three
621 different ways to evaluate the effects of hypobaric hypoxia *per sé* on changes in resting middle cerebral artery velocity.

	Term born	Preterm	P-value
$\Delta MCAv/\Delta S_aO_2$	3.1 ± 5.5	-0.2 ± 4.1	0.075
$\Delta MCAv/\Delta P_aCO_2$	1.5 ± 2.7	-0.1 ± 1.2	0.089
$\Delta MCAv/\Delta(P_aO_2/ P_aCO_2)$	-0.8 ± 1.8	0.4 ± 2.0	0.179

622 *Abbreviations*: MCA*v* = middle cerebral artery velocity; P_aCO_2 = arterial carbon dioxide partial pressure; P_aO_2 = arterial oxygen partial pressure;
623 S_aO_2 = arterial oxygen saturation.

Table 3. Cardiovascular and cerebrovascular responses to hypercapnia and hypocapnia at sea level and high-altitude (3375 m) in term born and preterm born participants.

	Sea level						High-altitude						Effect
	Term born			Preterm			Term born			Preterm			
	Baseline	*6%CO_2*	*Hypo*	*Baseline*	*6%CO_2*	*Hypo*	*Baseline*	*6%CO_2*	*Hypo*	*Baseline*	*6%CO_2*	*Hypo*	
HR (beats·min^{-1})	67 ± 9	65 ± 9	75 ± 11	69 ± 3	66 ± 5	71 ± 9	72 ± 2	69 ± 5	75 ± 7	70 ± 7	71 ± 5	76 ± 8	a,b
MAP (mmHg)	102 ± 9	108 ± 10	104 ± 10	96 ± 8	103 ± 9	98 ± 10	112 ± 14	113 ± 13	111 ± 12	111 ± 15	115 ± 14	113 ± 12	a,b
CVR*i* (mmHg·cm^{-1}·s^{-1})	1.91 ± 0.20	1.42 ± 0.16	2.38 ± 0.25	1.81 ± 0.48	1.51 ± 0.41	2.26 ± 0.53	2.04 ± 0.38	1.69 ± 0.30	2.66 ± 0.27	2.41 ± 0.61	1.81 ± 0.45	2.83 ± 0.73	a,b
CVC*i* (cm·s^{-1}·mmHg^{-1})	0.53 ± 0.05	0.71 ± 0.08	0.42 ± 0.05	0.58 ± 0.11	0.70 ± 0.15	0.46 ± 0.09	0.51 ± 0.11	0.61 ± 0.11	0.38 ± 0.04	0.44 ± 0.11	0.58 ± 0.14	0.38 ± 0.11	a,b
CrCP (mmHg)	27.1 ± 10.4	19.1 ± 11.5	38.9 ± 15.0	30.3 ± 12.9	22.2 ± 18.2	37.3 ± 19.4	15.4 ± 11.3	9.2 ± 15.8	24.6 ± 14.1	30.6 ± 13.8	21.0 ± 19.0	39.1 ± 13.1	b
RAP (mmHg·s^{-1}·cm^{-1})	1.06 ± 0.18	1.07 ± 0.31	1.20 ± 0.30	1.06 ± 0.51	0.98 ± 0.44	1.13 ± 0.60	1.37 ± 0.27	1.31 ± 0.21	1.49 ± 0.40	1.21 ± 0.29	1.31 ± 0.36	1.37 ± 0.46	a,b

Abbreviations: Baseline = ambient air; 6%CO_2 = hyperoxic hypercapnia; Hypo = voluntary hyperventilation to induce hypocapnia; HR = heart rate; MAP = mean arterial pressure; CVR*i* = cerebrovascular resistance index; CVC*i* = cerebrovascular conductance index; CrCP = critical closing pressure; RAP = resistance-area product. [a]P<0.05 main effect of altitude; [b]P<0.05 main effect of condition. No main or interaction effect involving the group factor was observed during any of the tested conditions.

Table 4. Arterial blood gas during sea level and high-altitude testing (3375 m) in term born and preterm born participants.

	Sea level						High-altitude						Effect
	Term born			Preterm			Term born			Preterm			
	Baseline	*6%CO_2*	*Hypo*	*Baseline*	*6%CO_2*	*Hypo*	*Baseline*	*6%CO_2*	*Hypo*	*Baseline*	*6%CO_2*	*Hypo*	
P_aO_2 (mmHg)	93 ± 7	470 ± 50	98 ± 5	92 ± 6	505 ± 48	96 ± 5	54 ± 1	311 ± 51	66 ± 6	55 ± 2	321 ± 40	67 ± 6	a,b,c
P_aCO_2 (mmHg)	42.4 ± 1.7	50.3 ± 3.1	29.8 ± 4.5	41.9 ± 2.9	49.7 ± 2.4	29.2 ± 5.2	33.9 ± 1.7	39.4 ± 1.8	26.0 ± 3.8	32.8 ± 2.3	39.4 ± 1.6	24.7 ± 4.5	a,b,c
pH	7.41 ± 0.01	7.36 ± 0.02	7.53 ± 0.04	7.41 ± 0.02	7.36 ± 0.03	7.52 ± 0.05	7.44 ± 0.02	7.40 ± 0.02	7.52 ± 0.04	7.45 ± 0.02	7.39 ± 0.02	7.53 ± 0.05	a,b,c,
$[H^+]$ (nM)	38.5 ± 1.0	43.3 ± 2.0	29.7 ± 3.0	39.0 ± 2.0	44.0 ± 3.0	30.2 ± 3.1	36.5 ± 1.7	40.1 ± 2.0	30.2 ± 2.7	35.7 ± 1.5	40.6 ± 1.6	29.8 ± 3.0	a,b,c
$[HCO_3^-]$ ($mEq \cdot L^{-1}$)	26.7 ± 1.4	28.2 ± 1.7	24.2 ± 1.8	26.2 ± 2.0	27.7 ± 2.0	23.5 ± 2.3	23.0 ± 1.2	24.3 ± 1.0	21.1 ± 1.7	22.6 ± 1.5	24.0 ± 1.1	20.3 ± 2.1	a,b
Base Excess ($mEq \cdot L^{-1}$)	2.0 ± 1.2	2.3 ± 1.5	2.4 ± 1.5	1.5 ± 2.0	1.5 ± 2.1	1.7 ± 1.9	-0.4 ± 1.1	-0.5 ± 1.1	0.2 ± 1.2	-0.4 ± 1.2	-0.9 ± 1.1	-0.2 ± 1.0	a,b
S_aO_2 (%)	97.2 ± 0.6	100.0 ± 0.0	99.0 ± 0.4	97.1 ± 0.5	100.0 ± 0.0	98.9 ± 0.4	89.3 ± 1.1	100.0 ± 0.1	95.5 ± 2.0	90.2 ± 1.7	99.9 ± 0.2	95.2 ± 1.7	a,b,c
[Hb] ($g \cdot dL^{-1}$)	16.6 ± 1.0	16.2 ± 0.8	16.6 ± 1.0	16.8 ± 1.8	16.5 ± 1.7	16.6 ± 1.9	17.5 ± 1.3	17.0 ± 1.0	17.1 ± 1.1	17.7 ± 0.9	17.5 ± 0.6	17.7 ± 0.6	a,b

Abbreviations: Baseline = ambient air; 6%CO_2 = hyperoxic hypercapnia; Hypo = voluntary hyperventilation to induce hypocapnia; P_aO_2 = arterial oxygen partial pressure; P_aCO_2 = arterial carbon dioxide partial pressure; $[H^+]$ = hydrogen concentration; $[HCO_3^-]$ = bicarbonate concentration; S_aO_2 = arterial oxygen saturation; [Hb] = haemoglobin concentration. $^aP<0.05$ main effect of altitude; $^bP<0.05$ main effect of condition; $^cP<0.05$ interaction effect of altitude and condition. No main or interaction effect involving the group factor was observed during any of the tested conditions.

Figure caption

Figure 1. Group means and individual values for pulmonary ventilation (upper panels) and end-tidal carbon dioxide partial pressure (lower panels) throughout the cerebrovascular CO2 reactivity protocol at sea level and at high-altitude in term born (blue circles) and preterm born (red circles) participants. Baseline = ambient air; 6%CO_2 = hyperoxic hypercapnia; Hypo = voluntary hyperventilation to induce hypocapnia.

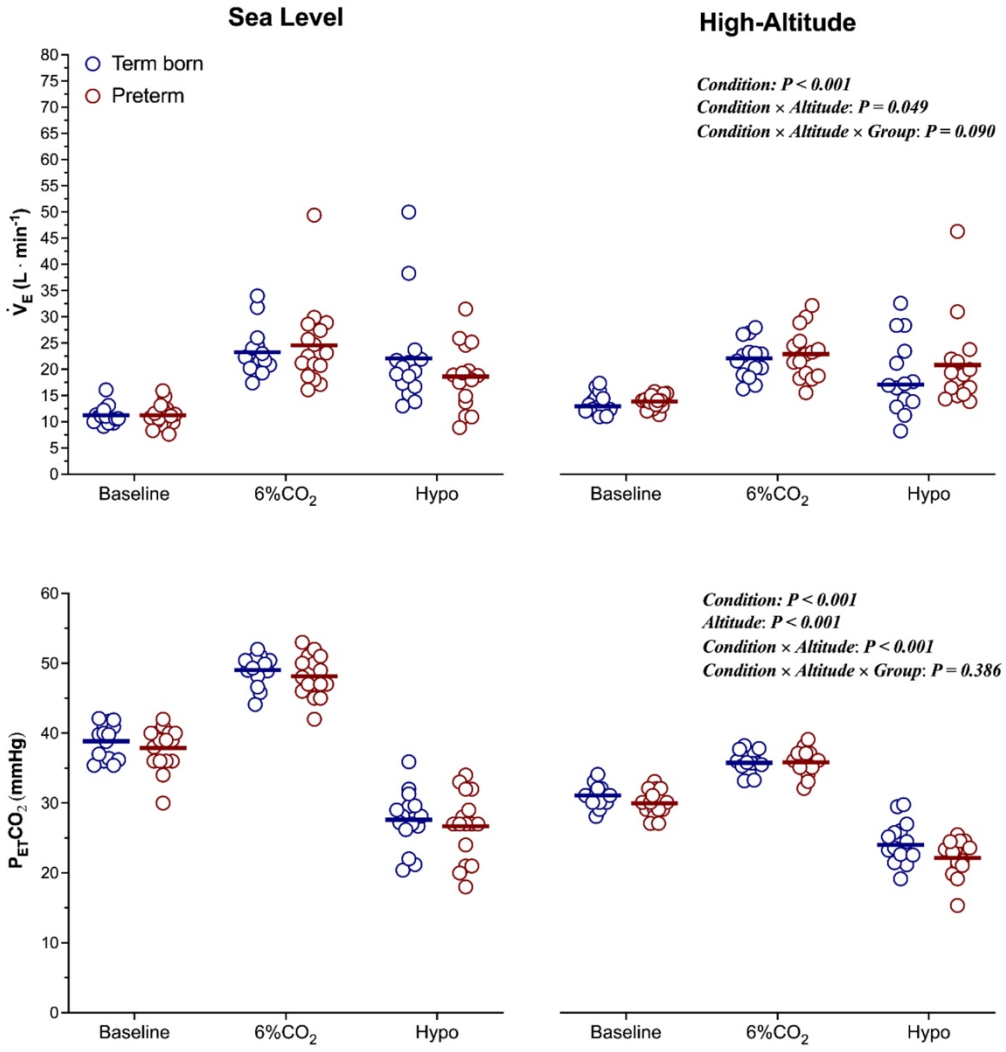
Figure 2. Representative examples of changes in middle cerebral artery velocity (MCAv) in response to changes in end-tidal carbon dioxide partial pressure ($P_{ET}CO_2$) in typical term born (upper panels) and preterm born (lower panels) participants at sea level (panels A and C, respectively) and at high-altitude (3375 m; panels B and D, respectively). The MCAv-$P_{ET}CO_2$ response was fit with a sigmoid curve (solid lines). The midpoint X_0 (black dot) of the sigmoid curve represents the optimal operating point of the vessels' capacity to dilate and constrict. Resting $P_{ET}CO_2$ represents baseline values of MCAv and $P_{ET}CO_2$ while breathing ambient air.

Figure 3. Group Mean (±SD) and individual values for middle cerebral artery velocity at sea level and at high-altitude (3375 m) while breathing ambient air (normoxic and hypoxic air, respectively) in term born (blue) and preterm born (red) participants.

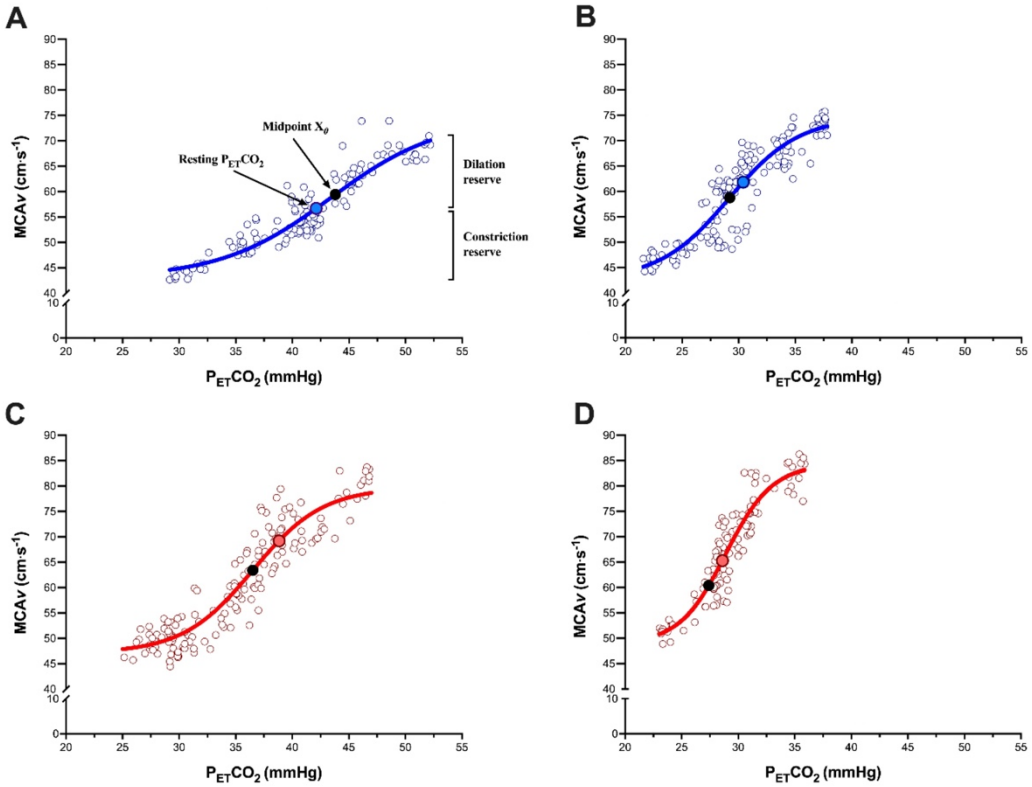
Figure 4. Hypercapnic and hypocapnic cerebrovascular reactivity at sea level (SL; circles) and at high-altitude (HH; 3375 m; triangles) in term born (blue) and preterm born (red) participants. Hypercapnic and hypocapnic cerebrovascular reactivity was determined as the percent change in middle cerebral artery velocity from baseline during a 4-min steady-state period breathing an hyperoxic hypercapnic gas mixture (6%CO_2 in 94%O_2) and a 4-min voluntary hyperventilation (to induce hypocapnia) phase, respectively, per unit change in end-tidal carbon dioxide partial pressure (%·$mmHg^{-1}$).

Figure 5. Group Mean (±SD) and individual values for cerebral vasodilation (panel A) and vasoconstriction (panel B) reserves at sea level and at high-altitude (3375 m) in term born (blue) and preterm born (red) participants.

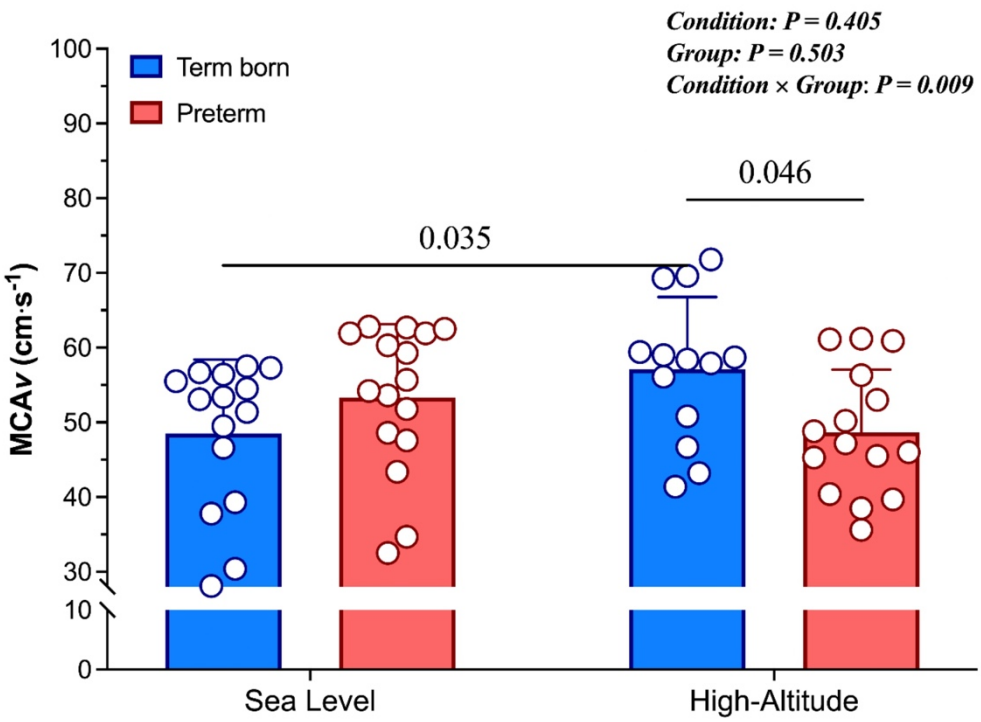
663 **Figure 1**


Sea Level
High-Altitude
Term born
Preterm
$\dot{V}_E$ (L · min^{-1})
Condition: P < 0.001
Condition × Altitude: P = 0.049
Condition × Altitude × Group: P = 0.090
Baseline
6%CO$_2$
Hypo
$P_{ET}CO_2$ (mmHg)
Condition: P < 0.001
Altitude: P < 0.001
Condition × Altitude: P < 0.001
Condition × Altitude × Group: P = 0.386

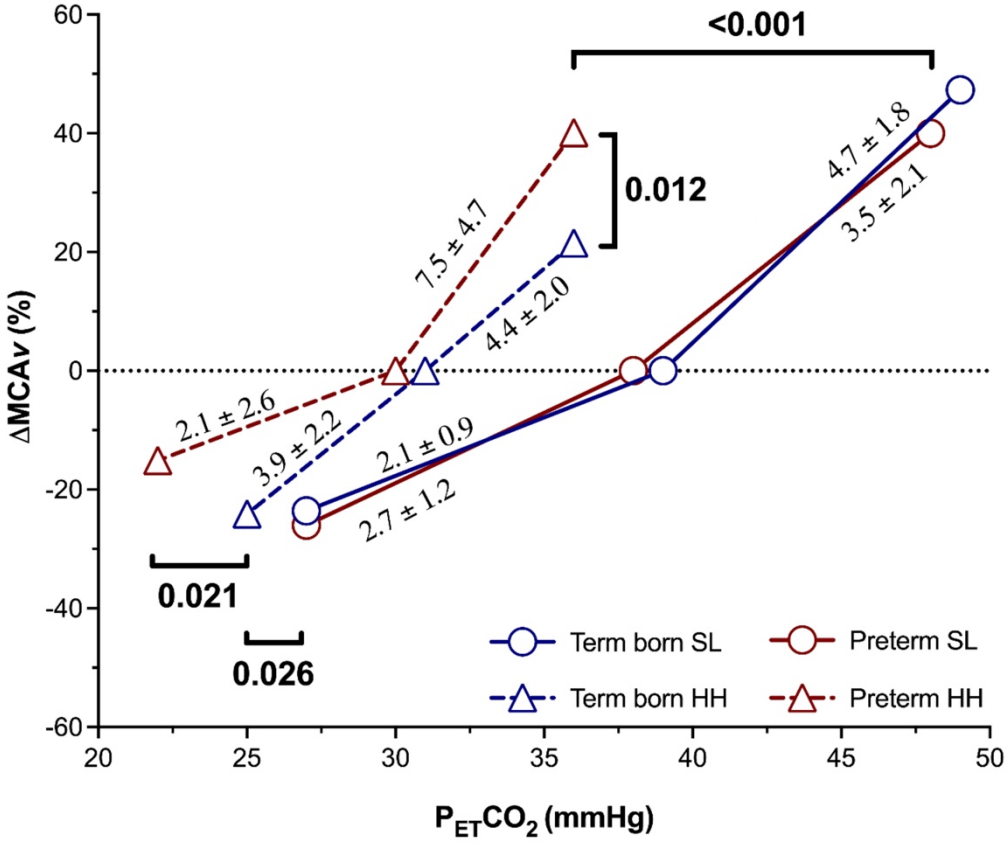
664 **Figure 2**


A
B
C
D
MCAv (cm·s⁻¹)
P_ETCO_2 (mmHg)
Midpoint X_0
Resting P_ETCO_2
Dilation reserve
Constriction reserve

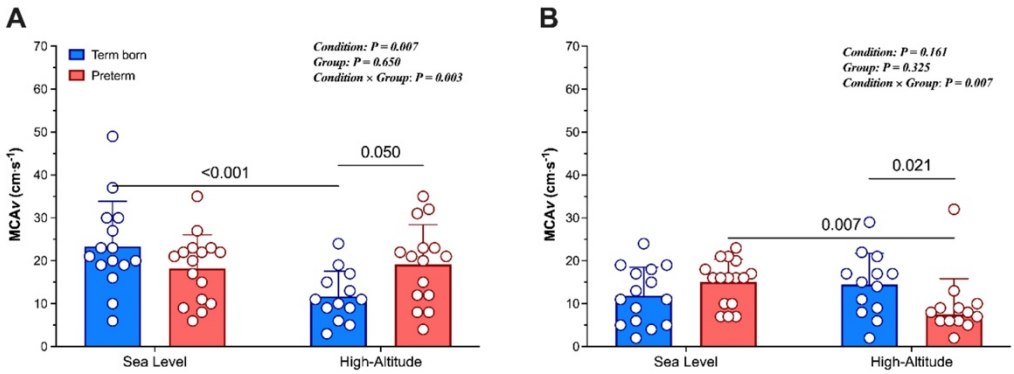
665 **Figure 3**


Condition: P = 0.405
Group: P = 0.503
Condition × Group: P = 0.009
Term born
Preterm
0.035
0.046
MCAv (cm·s-1)
100
90
80
70
60
50
40
30
10
0
Sea Level
High-Altitude

666 **Figure 4**


<0.001
0.012
7.5 ± 4.7
4.4 ± 2.0
2.1 ± 2.6
3.9 ± 2.2
2.1 ± 0.9
2.7 ± 1.2
4.7 ± 1.8
3.5 ± 2.1
0.021
0.026
Term born SL
Preterm SL
Term born HH
Preterm HH
ΔMCAv (%)
$P_{ET}CO_2$ (mmHg)
60
40
20
0
-20
-40
-60
20
25
30
35
40
45
50

667 **Figure 5**


A
Term born
Preterm
Condition: P = 0.007
Group: P = 0.650
Condition × Group: P = 0.003
MCAv (cm·s⁻¹)
<0.001
0.050
Sea Level
High-Altitude
B
Condition: P = 0.161
Group: P = 0.325
Condition × Group: P = 0.007
MCAv (cm·s⁻¹)
0.007
0.021
Sea Level
High-Altitude

13. Article 5

Oxidative stress and nitric oxide metabolism responses during prolonged high-altitude exposure in preterm born adults

Article 5 – Oxidative stress and nitric oxide metabolism responses during prolonged high-altitude exposure in preterm born adults

Chambion-Diaz M, Manferdelli G, Narang BJ, Giardini G, Debevec T, Pialoux V & Millet GP.

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Oxidative stress and nitric oxide metabolism responses during prolonged high-altitude exposure in preterm born adults

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Highlights

- In acute hypoxic exposure, preterm people present an increase antioxidant enzyme response and an inhibition of the pro-oxidant xanthine oxidase enzyme response.
- In prolonged hypoxic exposure, the antioxidant and pro-oxidant response return similar to the full-term subjects.
- This specific pro-oxidant response could be due a preconditioning phenomenon driven by the persistent imbalance of redox status after birth.

Abstract

Prematurely-born individuals tend to exhibit higher resting oxidative stress, although evidence suggests they may be more resistant to acute hypoxia-induced redox balance alterations. Given that oxidative stress responses to prolonged altitude exposure in this population have not yet been explored we, aimed to investigate the redox balance changes across a three-day hypobaric hypoxic exposure at 3375m in healthy adults born preterm (gestational age≤32 weeks) and term-born (gestational age≥38 weeks) counterparts.

Resting venous blood was obtained in normoxia (prior to altitude exposure), immediately upon arrival to altitude, and the following three mornings (D1-3). Antioxidant (superoxide dismutase, SOD; catalase; glutathione peroxidase, GPX and ferric reducing antioxidant power, FRAP), and pro-oxidant (xanthine oxidase, XO; myeloperoxidase, MPO) enzyme activity, oxidative stress markers (advanced oxidation protein products, AOPP; Malondialdehyde, MDA), NO metabolites (nitrites and nitrates, NOx), nitrotyrosine were measured in plasma.

SOD increased only in the preterm group ($p<0.05$). Catalase increased only at arrival in preterm group ($p<0.05$). GPX increased ($p<0.05$). XO activity increased only at D3 for the preterm group, while it only increased acutely (Arrival and D1) in control group. MPO increased in both groups through the 3 days ($p<0.05$). AOPP only increased at arrival in the preterm ($p<0.05$) whereas it decreased at the arrival up to D3 ($p<0.05$) for the control. MDA decreased in the control group from arrival onward. Nitrotyrosine decreased in both group ($p<0.05$). Nitrites increased on D3 ($p<0.05$) in the control group and decreased on D1 ($p<0.05$) in the preterm group.

These data indicate that antioxidant enzymes seem to increase immediately upon hypoxic exposure in preterm adults. Conversely, the blunted/shifted pro-oxidant enzyme response to prolonged hypoxia exposure suggests that these enzymes may be less sensitive in these preterm born. Overall, these findings lend further support to a potential hypoxic preconditioning effect of preterm birth.

Key words: hypoxia, premature birth, antioxidant, oxidative stress, altitude, nitrosative stress, prematurity

Abbreviations list:

AO antioxidant; **AOPP** advanced oxidation protein product; **FRAP** ferric reducing antioxidant power; **GPX** glutathione peroxidase; **H_2O_2** hydrogen peroxide; **PBMC** peripheral blood mononuclear cells; **MDA** malondialdehyde; **MPO** myeloperoxidase; **NO** nitric oxide; **NOx** total nitrite and nitrate; **NTB** nitrobluetetrazolium; **ROS** reactive oxygen species; **SOD** superoxide dismutase; **UA** uric acid; **XO** xanthine oxidase.

1. Introduction

According to the World Health Organization, 15 million babies are born preterm each year (before 37 completed weeks of gestation). The very premature babies (<32 weeks gestation) often present with cardiovascular and respiratory diseases or limitations [1]. These babies are also born with immature antioxidant defense systems, which may lead to further damage when they undergo oxygen therapy [2].

Oxidative stress is characterized by an imbalance between pro-oxidant and antioxidant enzyme activity, in favor of the former [3]. Oxidative stress can increase in response to several stimuli, including acute exercise and/or hypoxia. It is also known to be elevated in several acute and chronic diseases, as well as following premature birth [4]. Oxidative stress is characterized by the production of reactive oxygen species (ROS), mainly from the mitochondria, such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) [5]. The ROS can cause damage to proteins, fats and also DNA. To counteract the effect of ROS on cells, the organism presents an antioxidant defense system, characterized by enzymes or other components such as vitamins. However, in some pathophysiological conditions, this system can be immature or inefficient leading to ales effective protection against damages caused by ROS.

During premature birth, the newborn abruptly leaves the hypoxic intrauterine environment with an immature pulmonary system, and a lack of antioxidant defenses [6]. The baby then often undergoes pure oxygen therapy, which increases oxidative stress via the hypoxia reoxygenation redox pathway [4, 6]. However, it remains unclear whether this prematurity-related increase in oxidative stress observed at birth persists into adulthood. Recent findings indicate that adolescents born preterm exhibit higher oxidative stress measured by 8-isoprostane in exhaled breath, compared to age-matched adolescents born at term [6]. Consequently, the authors recommended limiting postnatal hyperoxic exposure as much as possible. On the contrary, no difference in oxidative stress biomarkers was observed between young adults born preterm and their peers born at term [7]. However, a lower birth weight for gestational age was associated with increased oxidative stress measured by urinary 8-isoprostane, suggesting that low birth weight may affect oxidative stress status in adult born preterm.

Acute hypoxic exposure is known to increase oxidative stress [8], and elicit rapid physiological responses, including the hypoxic ventilatory response [9, 10]. Many studies have reported an increase in oxidative stress markers after an acute hypoxic exposure in term-born

individuals [10, 11]. Usually, hypoxia-induced oxidative stress decreases NO bioavailability via NO inhibition by superoxide anion and form peroxynitrite ($ONOO^-$). However, prolonged hypoxia induces different physiological responses compared to acute hypoxia, in particular for oxidative stress and antioxidant capacity. At high altitude, hypoxia alters redox balance via ROS overproduction [12], also evidenced by increased oxidative stress markers in blood during a high-altitude sojourn [13]. Prolonged hypoxia also increases NO production and NO metabolites, eliciting beneficial effects on blood flow in healthy adults [14, 15], which are not observed in acute hypoxia. In addition, terrestrial altitude (hypobaric hypoxia) seems to induce higher levels of oxidative stress than simulated altitude (normobaric hypoxia), usually in hypoxic tent or chamber, most likely due to a larger hypoxic dose [16]. Moreover, the severity of the hypoxic hypobaric stimulus is likely greater than the normobaric hypoxic one at same partial pressure of O_2 [17].

As they are exposed to hypoxia early in their life, adults born preterm could be preconditioned to hypoxia, and this may limit ROS accumulation via greater antioxidant enzyme efficiency. In this context, Martin et al. showed that adults born preterm present a greater resistance to oxidative stress during hypoxic exercise, suggesting specific adaptations to hypoxia in this population [18]. This is likely due to their birth status and/or perinatal therapy. Moreover, in our recent work [18], we tested the hypothesis that prematurity would exacerbate exercise-induced oxidative stress, but mitigate hypoxia-induced oxidative stress. The hypoxic preconditioning leading to improved cellular antioxidant defenses is one of the hypotheses in favor of a decrease in oxidative stress in adults born preterm [18].

Since high altitude sojourns are becoming increasingly popular and accessible, improving our understanding of altitude-related oxidative stress modulation seems warranted. For full-term individuals, the oxidative stress responses to acute and prolonged hypoxic exposure are relatively well described. Conversely, for people born preterm, the acute oxidative stress response is beginning to be well characterized [18, 19], whereas the effects of prolonged or chronic exposure have not been investigated yet. Accordingly, we aimed to investigate the effects of prolonged exposure to hypobaric hypoxia on oxidative stress, antioxidant, and NO metabolite kinetics in prematurely born adults compared to a control group consisting of healthy, age- and aerobic capacity-matched full-term born individuals.

2. Materials & Methods

2.1 Participants

The experimental protocol was preregistered at ClinicalTrials.gov (NCT04739904), and ethical approvals were obtained from both the University of Ljubljana, Faculty of Sport ethics committee (8/2020-316), and the Aosta Hospital Ethical Committee (06/05/2021.0038781.I) The study was conducted in line with the guidelines of the Declaration of Helsinki. The preterm participants were recruited from the National Preterm Birth Register, managed by the University Clinical Centre in Ljubljana, Slovenia, using medical record screening and individual interviews. Preterm participants were required to fulfil the following inclusion criteria: gestational age ≤ 32 weeks, gestational mass ≤ 1500g, hyperoxic treatment at birth, lack of pulmonary diseases (i.e. bronchopulmonary dysplasia). Full-term adults had a gestational age of at least 38 weeks, and birth weight ≥2500 g. All participants were male and aged between 18 and 30 years. Participants were free from medical risk factors to exercise and/or altitude exposure, disease free, non-smokers, and had no exposure to altitude above 2000m for at least two months prior to participation. Details of baseline characteristic of the participants given in **Table 1** are also described elsewhere [20].

2.2 Experimental procedure

All participants were exposed to three days of hypobaric hypoxia (Torino hut, 3375 m, Mont Blanc massif, Italy). Blood samples were obtained from the antecubital vein at each of the following time points:

- Baseline - before traveling to altitude – resting values in normoxia.
- D0 - immediately upon arrival at altitude (2pm – 3pm)
- D1 - morning (fasting rested condition) sample following the first night at altitude.
- D2 - morning (fasting rested condition) sample following the second night at altitude.
- D3 - morning (fasting rested condition) sample following the third night at altitude.

Participants underwent various, but standardized exercise sessions during the three days at altitude.

2.3 Blood processing and Biochemical analyzes.

Blood samples were immediately centrifuged (10 min at 3500 rpm, 4°C) to separate the plasma, which was aliquoted into Eppendorf tubes which were stored at -20°C and -80°C until subsequent analysis. The following oxidative stress markers, pro-/anti-oxidants enzymes and NO metabolites were assessed: superoxide dismutase (SOD) and catalase activities, glutathione peroxidase (GPx) activity, the ferric reducing ability power (FRAP), the xanthine oxidase activity (XO), myeloperoxidase (MPO), the advanced oxidation protein product (AOPP), malondialdehyde (MDA), nitrites and nitrates (NOx), nitrotyrosine. All spectrophotometry measurements were performed with TECAN Infinite 2000 plate reader (Männedorf, Switzerland).

SOD

SOD is an antioxidant enzyme catalyzing the transformation of superoxide anion into hydrogen peroxide (H_2O_2). The activity of this enzyme is measured by the degree to which it inhibits the reaction between superoxides produced by the hypoxanthine-xanthine oxidase system, and the nitrotetrazolium blue (NTB), forming the formazan blue at 450 nm [21].

Catalase

Catalase allows the transformation of H_2O_2 into H_2O and O_2. Catalase activity was determined by measuring the kinetics (across 20 min) of formaldehyde apparition formed by the reaction between methanol and H_2O_2; a reaction also catalyzed by catalase. Absorbance is measured by a spectrophotometer at 540nm and compared to the formaldehyde range [22].

GPX

GPx activity was assessed by measuring nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) consumption, which is proportional to GPx activity to reduce H_2O_2 in the presence of glutathione reductase and reduced glutathione [23]. H_2O_2 is used as the initiator of the reaction. The kinetic was measured during 5 min by spectrophotometry at 340 nm.

FRAP

This antioxidant non-enzymatic marker reflects the ferric reducing antioxidant power. FRAP values were obtained by comparing the change in absorbance at 593 nm in samples, after

addition of ferric ion with ranges containing ferrous ions in known concentration [24]. An inverse correlation between FRAP and MDA has also been shown after hypoxic exposure [10], highlighting the potential sensitivity of FRAP to oxidative stress.

XO

XO catalizes the oxidation of hypoxanthine into xanthine and xanthine into uric acid. XO uses oxygen to create UA, but above all, superoxide anion. The xanthine oxidase activity was calculated by measuring the kinetic of appearance of the complex superoxide anion and nitrotetrazolium blue (NTB) by spectrophotometer at 560 nm [25].

MPO

MPO transforms H_2O_2 into hypochlorous acid (HOCl). As it crosses cell membranes, it can cause protein fragmentation and aggregation. The assay of MPO activity is described by *Suzuki et al.* [26] using TMB (tetramethylbenzidine), H_2O_2 to initiate the reaction. The kinetics of appearance of peroxidized TMB was measured by absorbance at 653 nm.

Plasmatic AOPP

AOPP is determined by the *Witko-Sarsat et al.* method [27], using a spectrophotometer. The result is expressed in chloramine-T equivalent, which, in the presence of potassium iodide (KI), absorbs at 340 nm.

Plasmatic MDA

As the end-product of polyunsaturated fatty acid oxidation, MDA is often used as an indicator of lipid peroxidation. The assay determines the MDA concentration in a sample from its reaction with thiobarbituric acid [28, 29]. A pink chromogen, formed by the MDA + 2TBA complex in an acidic and hot environment, is extracted using butanol. Its absorbance was measured at 532 nm with a spectrophotometer.

NOx / Nitrites

NO metabolites, nitrite and nitrate, were measured using Griess's reagent, a mixture of sulfanilamide, naphthalene-ethylene-diamine-dihydrochloride (NED) and phosphoric acid, by the modified method by *Bratton and Marshall* [30]. This reagent binds the nitrite to form a dye, which absorbs at 550 nm. In a second measure, nitrate reductase added to plasma sample was

used to convert nitrate into nitrite, thereby measuring the total amount of nitrites and nitrates (NOx).

Nitrotyrosine

The plasmatic concentration of nitrotyrosine was measured by ELISA as previously described [31]. Nitrotyrosine is an end product of protein nitration by the peroxynitrite, which is itself formed by the bond of superoxide anion and nitric oxide (NO).

2.4 Statistical analysis

Data are reported as mean ± SD. Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego, California, USA). Normality was assessed using the Shapiro-Wilk test. When normality was verified, a two-way mixed effects ANOVA was performed to compare the means of each assayed marker across time (Normoxia, A, D1, D2, D3) and between groups (Preterm, Full-term). In the event of a statistically significant main effect of time, or time*group interaction effect, a Fisher-LSD post-hoc test was used to locate statistically significant differences. The significance level was set at $p<0.05$.

3. Results

Data in A have been already published elsewhere [32]. For the full comprehension of the results, and to establish the complete time-course of the response to prolonged hypoxic exposure in our specific population, these data in A have been also included in the present study.

SOD did not change in the control group, whereas an increase was observed at D1 (+24%; p=0.0028) and D3 (+12%; p=0.0248) compared to normoxia in the preterm group (**Figure 1A**). Catalase did not change across the exposure in the control group (**Figure 1B**). A transient increase was however observed at A, relative to normoxia, in the preterm group (+58%; p<0.00001). GPx increased from A in both groups compared to normoxia (+6.5% for control group p=0.0011 and +12% for preterm group p=0.0002). Compared to normoxia, FRAP decreased in both the control group (-15%; p=0.0004) and the preterm group (-8%; p=0.0082) at D1.

XO increased relative to A in the control group (+124% vs normoxia, p=0.0028), and remained elevated relative to normoxia until D3 (**Figure 1C**). In the preterm group, XO

increased only at D3, relative to normoxia (+65%; p=0.0235). MPO increased in both groups across the three days (+31% at D3 vs normoxia, p=0.0051 for control and +26.6% at D3 vs normoxia, p=0.0041 for preterm).

AOPP decreased at A in the control group (-25%; p=0.0218), and remained lower until D2 (-11.4%; p=0.0277). In preterm, AOPP was elevated only at A (+28%, p=0.0444) (**Figure 1D**). MDA decreased in the control group compared to normoxia from A to D3 (-17%; p=0.0005). In contrast, MDA decreased at A and returned to baseline from D1 in the preterm group.

Nitrites increased in D3 (+42%; p=0.0166) compared to D1 in the control group, while a decrease was seen at D1 (-41%; p=0.0091) in the preterm group. Nitrotyrosine decreased only in D3 (-25%; p=0.0247) in the control group compared to A, whereas a decrease at D2 (-21%; p=0.0013) was observed in the preterm group (Error! Reference source not found.).

4. Discussion

The aim of the present study was to investigate the effects of prolonged high-altitude exposure on oxidative stress markers, pro-/anti-oxidants enzyme activity, and NO metabolite kinetics in healthy adults born preterm, and age- and exercise capacity-matched controls. This is the first study to investigate these responses throughout several days of hypobaric hypoxic exposure in this population.

Adults born preterm expressed unique oxidative stress dynamics compared to the control participants. Indeed, an acute increase in antioxidant enzyme activity (SOD and catalase) and the protein oxidation marker AOPP was observed in the preterm group, although these returned to baseline levels at D1 or D2. There was no significant change in the control group for these markers. Conversely, the pro-oxidant enzyme XO activity increased only at the end of the prolonged hypoxia exposure (D3) for the preterm, while it only increased acutely (i.e., arrival and D1) in the control group. These differences in oxidative stress kinetics in response to prolonged hypoxia suggests specific redox balance modulation in prematurely-born adults.

In the preterm group, SOD and catalase increased immediately upon arrival at high-altitude, whereas there was no change in the control group. This suggests that preterm individuals likely have a higher oxidative stress response to hypoxia than the control group,

possibly due to increased radical production (superoxide and H_2O_2). SOD and catalase were increased during prolonged hypoxia in healthy term-born participants [33]. The following days, the AOPP levels decreased likely due to an augmentation of antioxidant enzymes activity. On the contrary to SOD and catalase, our results show that the increase of the pro-oxidant enzyme XO activity is delayed in the preterm compared to controls. It appears therefore that the hypoxic stimulus duration and/or intensity was insufficient for prematurely-born participants to stimulate XO. On the other hand, this could also suggest that the XO pathway may be less sensitive to hypoxic stress in the preterm participants. This AOPP increase only at arrival in preterm subjects was however not explained by the XO activity (increase only in D3) or MPO activity (similar in both groups).

Other redox mechanisms generating superoxide such as NADPH oxidase activation or NOS uncoupling could be involved in this specific response to hypoxic exposure [34]. The higher radical production could also originate in the mitochondria; i.e., Kumari et al. [35] reported that mitochondrial respiration in the peripheral blood mononuclear cells of prematurely-born people individuals was greater than in a full-term group. This should consequently reduce ROS production in these individuals [36, 37]. Further experiments seem warranted to confirm these hypotheses. An increase in GPX activity from A to D3 was seen regardless of birth status in this study, contradicting the results of Martin et al. [38]. The discrepancy between the GPX and catalase kinetics, given that both reduce H_2O_2, may be due to a higher affinity and lower scavenging efficiency of GPX to H_2O_2. Under conditions of high H_2O_2 production, as observed in hypoxia in preterm individuals, the hypoxia-induced prooxidant stimulus could be sufficient to overwhelm GPX and thus stimulate catalase [39, 40].

In hypoxia, MPO catalyzes the formation of hypochlorous acid. This acid can cross cell membranes and cause protein fragmentation and subsequent oxidative stress [41]. The increase from D1 to D3 in MPO activity in both groups suggests that premature birth might not influence mechanisms in hypoxia with no specific adaptation as seen in SOD, catalase, and XO. For the lipid oxidation marker MDA, the same kinetics were also observed in both groups. The decrease in MDA in preterm and full-term individuals may be the result of increased GPX activity. These results however contrast with AOPP, although this could be explained by their different production pathways. MDA is the end-product of polyunsaturated fatty acid peroxidation. Plasma MDA is also a reflection of whole-body rich-PUFA oxidation, and also has low sensitivity to pro-oxidant stimuli (Lefevre et al., 1998). Plasma AOPP is primarily the result of advanced protein oxidation by ROS formed via myeloperoxidase reaction during

oxidative/chlorine stress [27]. Regarding NO metabolites, no specific differences were observed during prolonged altitude exposure between the groups for nitrites. This contrasts previous studies which have reported that people born preterm could present attenuated NO metabolism in acute hypoxia [38]. The nitrites decrease in D1 in both groups is certainly due to radical overproduction limiting NO bioavailability [42, 43].

The XO kinetics on the one hand and SOD and catalase on the other hand, suggest that adults born preterm may present a hypoxic preconditioning state, at least in relation to oxidative stress [38]. Indeed, the prooxidant activity of XO appears to be less sensitive to a hypoxia stimulus, whereas the antioxidant activity of SOD and catalase seems transiently upregulated in preterm individuals as an acute burst of ROS production upon initial exposure to hypoxia. We speculate that this prooxidant/antioxidant specific response among preterm-born adults may be an hermetic adaptation [34]. Specifically, while neonatal oxidative stress may lead to initial detrimental effects, chronic ROS production may promote adaptation across the lifespan that may even exceed normal function [44]. Because of their immature biological systems, premature newborns were required to adapt to their environment as a survival mechanism. The persistent imbalance in redox status after birth could lead to a state of "preconditioning" to oxidative stress later in their lives [18]. More specifically, adults born preterm may have developed physiological adaptative mechanisms to hypoxia aiming to cope with large variations in oxygen experienced at birth and known to increase the ROS production. The understanding of these underlying specific modulations of oxidative stress requires further investigations.

5. Limitations

Despite its novelty, the present study has some limitations that we would like to acknowledge. First, MDA is criticized as a marker of lipid peroxidation as it is rapidly metabolized by the liver [45]. Markers more sensitive and more specific to lipid peroxidation exist, such as isoprostanes whose assays are based on chromatography techniques associated with mass spectroscopy [46]. However, although less sensitive, the hypoxic stimulus is considered strong enough to observe changes in MDA concentrations.

There are also limitations related to the field testing at high altitude. While the exercise testing performed during the sojourns might have modulated the observed oxidative stress differences [32], it is important to emphasize that these tests were standardized and conducted at the same time points for all the participants. Additionally, this study only focused on male

297 participants, so we cannot directly extend the results to female participants. In particular,
298 hormonal changes throughout the menstrual cycle are known to influence oxidative stress [47],
299 exercise [48], and hypoxic responses [49].

6. Conclusion

300 This is the first study to examine the effects of prolonged hypoxic exposure in a sample
301 of prematurely born, but otherwise healthy, adults, on the kinetics of oxidative stress,
302 antioxidants markers and NO metabolism. It appears that the preterm born individuals exhibit
303 different oxidative stress and antioxidant responses to prolonged hypoxia compared to their
304 term born counterparts. These specific oxidative stress responses may be the result of long-term
305 hypoxic preconditioning. Further research seems warranted to establish the underlying
306 mechanisms through which preterm birth (and associated treatments) may induce the results
307 observed in this study.

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Author contribution statement

G.P.M. and T.D. conceived the research and obtained the financial support. G.M., B.J.N., G.G., T.D., and G.P.M. contributed to the experimental design. G.M. and B.J.N. collected the data. M.C.D and V.P. analyzed the plasma samples for oxidative stress markers, antioxidant enzymes, and nitric oxide metabolites. M.C.D., G.M., V.P., and G.P.M. analyzed and interpreted the data. M.C.D. drafted the manuscript. All authors critically revised the draft and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1. Characteristics of the participants. Data are presented as mean ± SD.

	Full-term	Preterm	P
N	17	17	
Gestational age (weeks)	40 ± 0	29 ± 1	<0.001
Birth weight (g)	3621 ± 101	1132 ± 64	<0.001
Age at test (years)	21 ± 1	21 ± 1	0.066
Height (m)	1.82 ± 2	1.78 ± 2	0.210
Body mass (kg)	75.6 ± 1.7	72.4 ± 3.5	0.415
BMI ($kg.m^{-2}$)	22.8 ± 0.4	22.5 ± 0.7	0.713
$\dot{V}O_2$peak ($ml.kg^{-1}.min^{-1}$)	51.9 ± 1.9	48.5 ± 2.6	0.290
FVC (L)	5.67 ± 0.17	5.37 ± 0.31	0.285
FVC (%predicted)	98 ± 2	98 ± 3	0.809
FEV_1 (L)	4.63 ± 0.18	4.23 ± 0.13	0.082
FEV_1 (% predicted)	93 ± 2	92 ± 2	0.412

Abbreviations: *BMI*, body mass index; *FEV_1*, forced expired volume in 1sec; *FVC*, forced vital capacity; *MVV*, maximal voluntary ventilation; *PEF*, peak expiratory flow; *$\dot{V}O_{2peak}$*, peak oxygen uptake.

Table 2. Plasmatic concentration of oxidative stress markers, nitrites, and Nitrotyrosine in full-term and preterm participants, in normoxia and during three days of high-altitude exposure (3375 m). Data are expressed as mean ± SD.

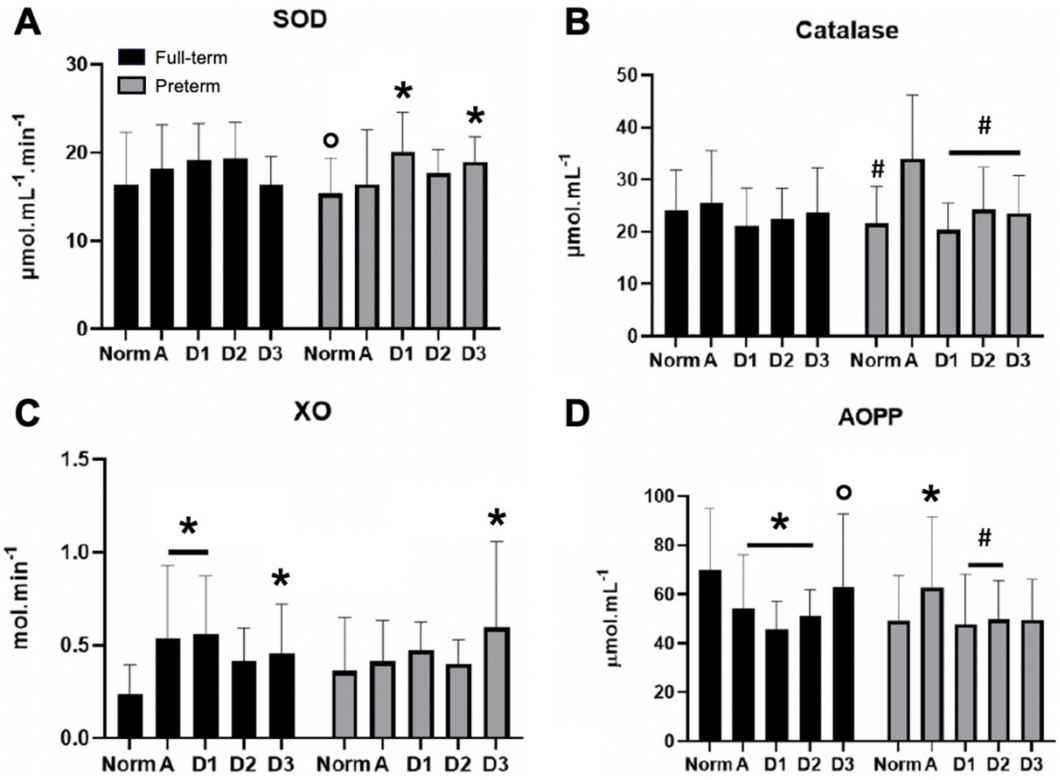
	Full-term					Preterm				
	Norm	A	D1	D2	D3	Norm	A	D1	D2	D3
MPO $mmol.L^{-1}.min^{-1}$	7.9 ± 2.6	9.5 ± 2.7°	11.2 ± 1.7*	10.5 ± 2.6*°	10.3 ± 2.7*	9.7 ± 2.0	10.5 ± 2.2	11.2 ± 1.3	11.8 ± 2.2*	12.2 ± 1.6*
MDA $\mu mol.mL^{-1}$	9.0 ± 0.8	7.9 ± 1.0*	8.0 ± 0.5*	7.6 ± 0.8*	7.5 ± 0.7*	8.5 ± 1.5	7.0 ± 1.1*	8.1 ± 0.5#	8.1 ± 0.7#	7.6 ± 1.5*
FRAP $mmol.L^{-1}$	649 ± 108	574 ± 113	529 ± 48*	528 ± 83*	569 ± 82*	586 ± 80	639 ± 141	533 ± 60#	553 ± 102	561 ± 98#
GPX $\mu mol.mL^{-1}.min^{-1}$	10.5 ± 1.2	11.9 ± 1.0*	11.5 ± 1.4*	12.4 ± 1.3*	11.2 ± 1.6¤	10.5 ± 1.1	11.9 ± 1.0*	11.8 ± 0.9*	11.9 ± 0.9*	11.5 ± 1.0*
Nitrites $\mu M.mL^{-1}$	12.1 ± 6.5	12.9 ± 6.5	10.3 ± 4.0$	11.4 ± 4.8	15.7 ± 10.6	12.4 ± 7.0	15.3 ± 9.2°	7.3 ± 3.1*	12.1 ± 6.4°	11.3 ± 3.2
Nitrotyrosine $\mu mol.mL^{-1}$	12.1 ± 4.6	12.0 ± 3.9$	12.0 ± 2.0$	11.9 ± 2.8	9.0 ± 3.3	12.2± 5.1¤	11.6 ± 4.4	12.3 ± 3.7¤	10.3±2.5	10.3 ± 3.3°

Abbreviations: *MPO*, myeloperoxidase; *MDA*, malondialdehyde; *FRAP*, ferric reducing antioxidant power; *GPX*, glutathione peroxidase; *Norm*, normoxia; *A*, arrival at high-altitude; *D1-D2-D3*, day 1-2-3 at high-altitude. $p<0.05$ vs Norm: *; $p<0.05$ vs Arrival: #; $p<0.05$ vs D1: °; $p<0.05$ vs D2: ¤; $p<0.05$ vs D3: $.

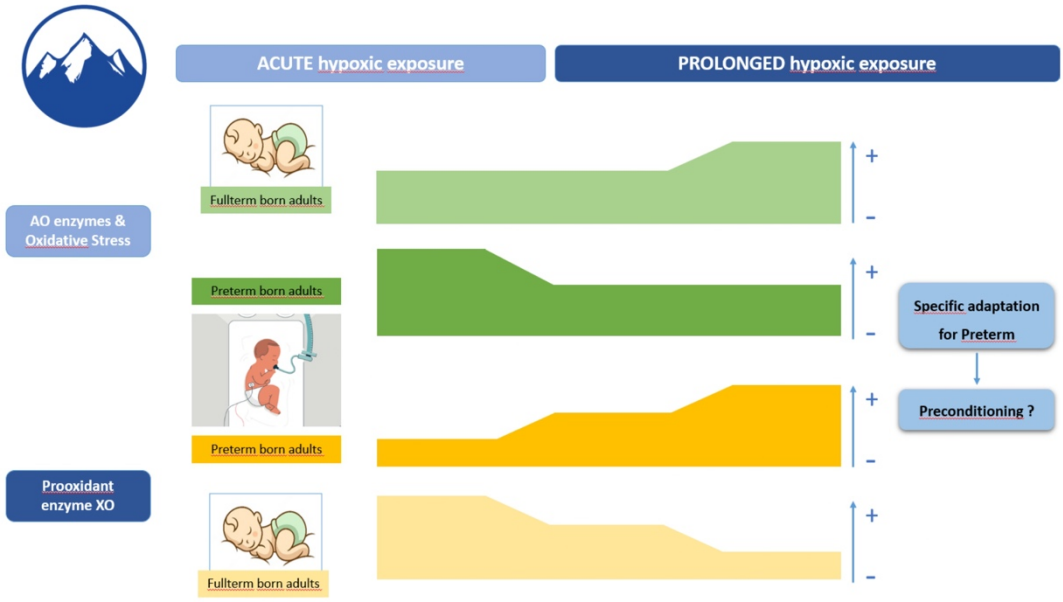
Figure captions

Figure 1. Plasmatic concentration of advanced oxidation protein product (AOPP - Panel A), xantine oxidase (XO) activity (Panel B), superoxide dismutase (Panel C), and catalase activity (Panel D) in the full-term (control - dark bars) and preterm (light grey bars) participants at each timepoint. $p<0.05$ vs Normoxia: *; $p<0.05$ vs Arrival: #; $p<0.05$ vs D1: °; $p<0.05$ vs D2: ¤; $p<0.05$ vs D3: $.

Figure 1


A
SOD
Full-term
Preterm
µmol.mL-1.min-1
30
20
10
0
Norm A D1 D2 D3 Norm A D1 D2 D3
B
Catalase
µmol.mL-1
50
40
30
20
10
0
Norm A D1 D2 D3 Norm A D1 D2 D3
C
XO
mol.min-1
1.5
1.0
0.5
0.0
Norm A D1 D2 D3 Norm A D1 D2 D3
D
AOPP
µmol.mL-1
100
80
60
40
20
0
Norm A D1 D2 D3 Norm A D1 D2 D3

Graphical abstract



"Fail big, today is the beginning of the rest of your life and you can be very frightening. And it's a new mean world out there. You only live once, so do what you feel passionate about. Take chances professionally, don't be afraid to fail, […] don't be afraid to go outside the box. Don't be afraid to think outside the box. Don't be afraid to fail big, to dream big, but remember, dreams without goals, are just dreams.

So have dreams, but have goals, life goals, yearly goals, monthly goals, daily goals. […] and understand that to achieve these goals you must apply discipline and consistency.

We don't plan to fail, we fail to plan – hard work works, working really hard is what successful people do."

Denzel Washington