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Exaggerated systemic oxidative-inflammatory-nitrosative stress in chronic mountain sickness is associated with cognitive decline and depression

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Key points

- Chronic mountain sickness (CMS) is a maladaptation syndrome encountered at high altitude (HA) characterised by severe hypoxaemia that carries a higher risk of stroke and migraine and is associated with increased morbidity and mortality.
- We examined if exaggerated oxidative-inflammatory-nitrosative stress (OXINOS) and corresponding decrease in vascular nitric oxide bioavailability in patients with CMS (CMS+) is associated with impaired cerebrovascular function and adverse neurological outcome.
- Systemic OXINOS was markedly elevated in CMS+ compared to healthy HA (CMS−) and low-altitude controls.
- OXINOS was associated with blunted cerebral perfusion and vasoreactivity to hypercapnia, impaired cognition and, in CMS+, symptoms of depression.
- These findings are the first to suggest that a physiological continuum exists for hypoxaemia-induced systemic OXINOS in HA dwellers that when excessive is associated with accelerated cognitive decline and depression, helping identify those in need of more specialist neurological assessment and targeted support.

Damian M. Bailey embarked on a PhD in human physiology while working as a research physiologist at the British Olympic Medical Centre in collaboration with Oxford University. Following training at the Universities of California San Diego and Colorado Health Sciences Center, he returned to the University of South Wales where he is currently Professor of Physiology & Biochemistry and a Royal Society Wolfson Research Fellow leading the Neurovascular Research Laboratory. His research programme takes an integrated translational approach to investigate how free radicals and associated reactive oxygen/nitrogen species control oxygen delivery to the brain across the clinical spectrum of human health and disease.

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Abstract Chronic mountain sickness (CMS) is a maladaptation syndrome encountered at high altitude (HA) characterised by severe hypoxaemia that carries a higher risk of stroke and migraine and is associated with increased morbidity and mortality. The present cross-sectional study examined to what extent exaggerated systemic oxidative-inflammatory-nitrosative stress (OXINOS), defined by an increase in free radical formation and corresponding decrease in vascular nitric oxide (NO) bioavailability, is associated with impaired cerebrovascular function, accelerated cognitive decline and depression in CMS. Venous blood was obtained from healthy male lowlanders (80 m, $n = 17$), and age- and gender-matched HA dwellers born and bred in La Paz, Bolivia (3600 m) with (CMS+, $n = 23$) and without (CMS−, $n = 14$) CMS. We sampled blood for oxidative (electron paramagnetic resonance spectroscopy, HPLC), nitrosative (ozone-based chemiluminescence) and inflammatory (fluorescence) biomarkers. We employed transcranial Doppler ultrasound to measure cerebral blood flow (CBF) and reactivity. We utilised psychometric tests and validated questionnaires to assess cognition and depression. Highlanders exhibited elevated systemic OXINOS ($P < 0.05$ *vs.* lowlanders) that was especially exaggerated in the more hypoxaemic CMS+ patients ($P < 0.05$ *vs.* CMS−). OXINOS was associated with blunted cerebral perfusion and vasoreactivity to hypercapnia, impaired cognition and, in CMS+, symptoms of depression. Collectively, these findings are the first to suggest that a physiological continuum exists for hypoxaemia-induced OXINOS in HA dwellers that when excessive is associated with accelerated cognitive decline and depression, helping identify those in need of specialist neurological assessment and support.

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Introduction

The human brain has evolved a much higher rate of obligatory oxygen (O_2) consumption because, unlike most other organs, its evolutionary 'drive for size' means that the brain is committed to a continually active state, demanding a disproportionate 20% of the body's basal O_2 budget in the resting state, more than 10 times that expected from its mass alone (Bailey *et al.* 2017*b*). The requirement to process large amounts of O_2 over a relatively small tissue mass supports the high rate of ATP formation to fuel the maintenance of ionic equilibria and uptake of neurotransmitters for synaptic transmission (Alle *et al.* 2009). That cognitive function is impaired by acute hypoxia is thus not surprising and an extensive literature has since documented proportional deficits in executive function, attention, mental speed, language and memory (Virues-Ortega *et al.* 2004; Rimoldi *et al.* 2016; McMorris *et al.* 2017), including lasting damage to white and grey matter motor architecture (Di Paola *et al.* 2008).

However, few studies have considered how lifelong exposure to hypoxia affects cognitive function and whether compensatory adaptations to sustain adequate tissue O_2 delivery prevent acute impairments from potentially progressing to irreversible dementia. The lack of information is surprising given an estimated 140 million high-altitude (HA) dwellers permanently live above 2500 m through economic and social necessity (Bailey *et al.* 2018*b*) and an evolving body of literature indicating that hypoxaemia is responsible for the higher prevalence of cognitive impairment and dementia observed in patients living at sea level with cardiopulmonary disease (Peers *et al.* 2009; Bagge *et al.* 2018).

In the few studies published to date, cognitive function has been shown to be slightly impaired in well-adapted elderly Andean HA dwellers relative to sea-level-based lowlander controls (Yan *et al.* 2011; Hill *et al.* 2014; Davis *et al.* 2015*b*). In contrast, albeit in the only study conducted to date, the authors observed an inverse relationship between altitude of residence and (age-adjusted) dementia mortality rate. However, this study examined patients living at considerably lower altitudes (up to 1800 m) in California with no control of potential confounders such as comorbidities and air pollution, highlighting the need for additional studies.

Furthermore, the mechanism underpinning altitude-induced cognitive impairment is unclear and to what extent further impairments occur in highlanders suffering from chronic mountain sickness (CMS+), a maladaptation syndrome characterised by exaggerated erythrocytosis and more pronounced hypoxaemia (Villafuerte & Corante, 2016), has not been examined. We have previously identified that compared to lowlander

(normoxic) controls, systemic oxidative–nitrosative stress (OXNOS), defined by an increase in free radical formation and corresponding decrease in vascular nitric oxide (NO) bioavailability, was permanently elevated in healthy well-adapted Andeans living at 3600 m without CMS (CMS−). The observation that systemic vascular endothelial function remained intact suggested that physiological concentrations of OXNOS may prove hormetically beneficial for lifelong adaptation to the hypoxia of HA. In contrast, highlanders with CMS (CMS+) exhibited more exaggerated increases in OXNOS and impaired systemic vascular endothelial function, thus implying a potential metabolic basis to HA maladaptation (Bailey *et al.* 2013*c*).

In light of these findings, we sought to determine the potential relationships between metabolic (oxidative–inflammatory–nitrosative stress, hereafter OXINOS), haemodynamic (cerebrovascular function) and clinical (cognition and depression) correlates in CMS+ and CMS− in order to provide more integrated mechanistic insight into the potential pathophysiology and consequences of neurological maladaptation to HA. We hypothesised that compared to lowlander controls, systemic OXINOS would be moderately elevated in CMS− in the absence of any impairments in cerebrovascular function, cognition or symptoms of depression. We further hypothesised that the systemic OXINOS response would be further exaggerated in CMS+ subsequent to more pronounced arterial hypoxaemia and associated with impairments in cerebrovascular function, cognition and symptoms of depression. Figure 1 provides a schematic summary of the proposed mechanisms.

Materials and methods

Ethical approval

The experimental protocol was approved by the Institutional Review Boards for Human Investigation at the University of San Andres, La Paz, Bolivia (CNB #52/04), University of Lausanne, Lasusanne, Switzerland (#89/06, #94/10), and University of Glamorgan, Pontypridd, UK (#4/07), and subsequently registered (clinicaltrials.gov; Identifier: NCT01182792). All participants were informed of the purpose/risks of the experiment and signed an informed consent form, with all procedures adhering to guidelines set forth in the *Declaration of Helsinki*.

Experimental design

The study was a cross-sectional population-based observational study in accordance with the STROBE statement (von Elm *et al.* 2014). Figure 2 provides a schematic of the experimental design. The Neurovascular Research Laboratory in the UK (∼80 m) was the site of investigation for the lowlanders and the Instituto Boliviano de Biologia de Altura in La Paz, Bolivia (∼3600 m) for the highlanders.

Participants

For all participants, inclusion criteria specified that they were born and had lived permanently at their resident altitude and were sedentary (defined as no formal

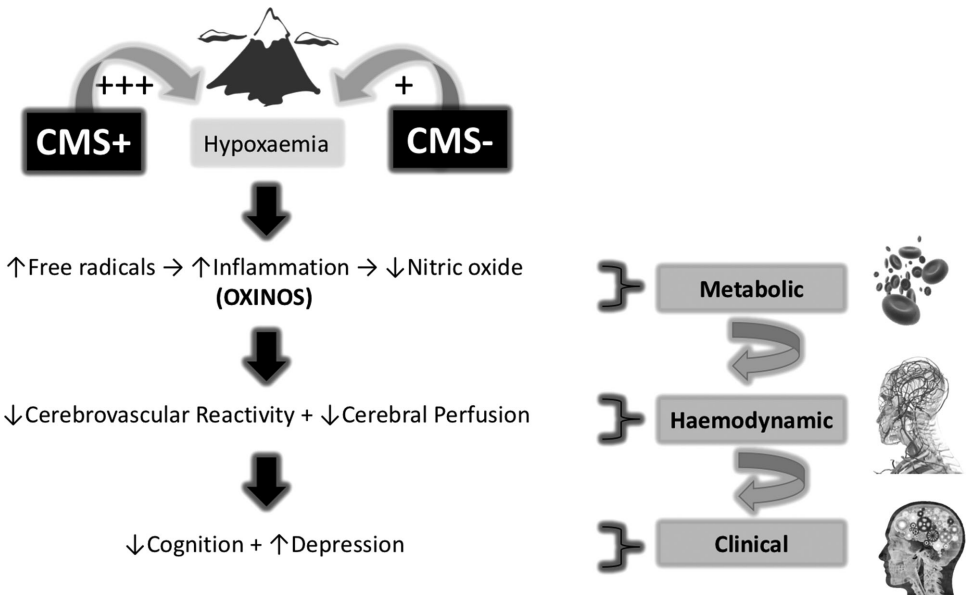

Figure 1. Functionally integrated translational hypothesis
CMS−/CMS+, highlanders without/with chronic mountain sickness; +++, comparatively more hypoxaemic; OXINOS, oxidative–inflammatory–nitrosative stress.

recreational activity outside of everyday living, Bailey *et al.* 2013*b*). Exclusion criteria included those with significant developmental delay or learning difficulties, diagnosis of any central neurological disease such as aneurysm, stroke, transient ischaemic attack, epilepsy, multiple sclerosis and psychiatric disorders including any history of traumatic brain injury and hypertension. None of the participants was taking nutritional supplements including over-the-counter antioxidant or anti-inflammatory medications. We specifically chose to exclude females given our inability to control for differences in circulating oestrogen known to affect cerebral blood flow (CBF) and cognition (Yao *et al.* 2009). Before inclusion into the study, all participants were subject to an extensive clinical examination that consisted of a thorough medical history, chest auscultation and 12-lead ECG. Participants were subsequently familiarised with the equipment and procedures.

Highlanders. We recruited 23 male patients with primary CMS (CMS+) and 14 healthy age-, gender- and education- (consecutive years in secondary and University education) matched controls without CMS (CMS−) native to La Paz, Bolivia (Table 1). We scored symptoms of CMS and confirmed clinical diagnosis by an excessive erythrocytosis [haemoglobin (Hb) $>$ 20 g/dL] in the presence of normal pulmonary function and no history of working in the mining industry (Leon-Velarde *et al.* 2005). All participants identified themselves as Aymaras and were from similar socio-economic backgrounds having been born and bred in La Paz with Spanish spoken as their first language.

Lowlanders. We also recruited 17 age- and education-matched healthy Caucasian males born and bred close to sea-level (∼80 m) in the UK (Table 1) as a sea-level (normoxic) comparator.

Educational status

Data were collected on level of full-time education attained.

Metabolic assessments

Participants were asked to refrain from physical activity, caffeine and alcohol and to follow a low nitrate/nitrite (NO_3^-/NO_2^-) diet 24 h prior to formal experimentation (Woodside *et al.* 2014) and were subject to a 12 h overnight fast when they attended the laboratory at 08.00 h. We obtained blood samples without stasis following 20 min of seated rest to control for plasma volume shifts.

Chemicals. All chemicals were of the highest available purity from Sigma-Aldrich (Poole, UK).

Blood sampling. We collected blood from an indwelling cannula located in a forearm antecubital vein into Vacutainers (Becton, Dickinson and Company, Oxford, UK) before centrifugation at 600 *g* (4°C) for 10 min. We decanted plasma and serum samples into cryogenic vials (Nalgene Labware, Thermo Fisher Scientific Inc., Waltham, MA, USA), that were immediately snap-frozen in liquid nitrogen (N_2) and shipped/stored under N_2 gas (Cryopak, Taylor-Wharton, Theodore, AL, USA) before analysis in the UK. We left samples to defrost at 37°C in the dark for 5 min before batch analysis.

Oxidative stress.

Antioxidants. We assayed plasma concentrations of reduced and oxidised glutathione (GSH/GSSG) according to the methods established by N'Guessan *et al.* (2011) with modifications (Stocker *et al.* 2017). Intra- and inter-assay coefficients of variation (CVs) were both $<$5%.

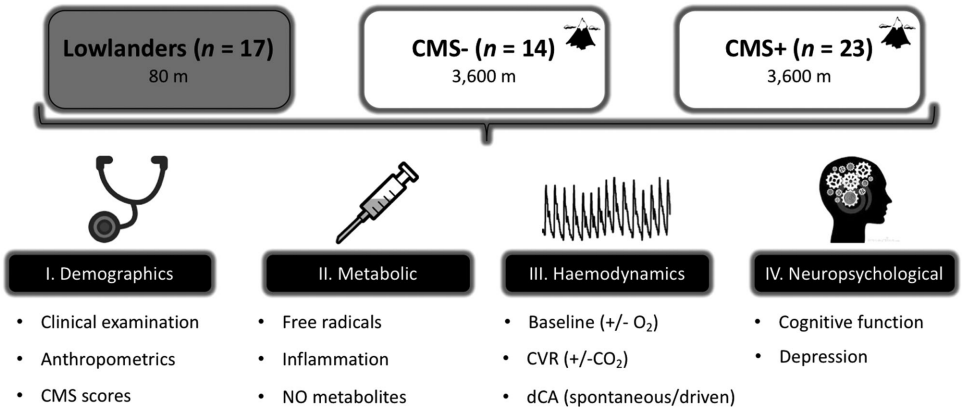

Figure 2. Experimental design
CMS−/CMS+, highlanders without/with chronic mountain sickness; NO, nitric oxide; O_2, oxygen; CVR, cerebrovascular reactivity; CO_2, carbon dioxide; dCA, dynamic cerebral autoregulation.

Table 1. Demographic data

Group:	Lowlanders	Highlanders		*P* values		
Subgroup:	Controls ($n = 17$)	CMS− ($n = 14$)	CMS+ ($n = 23$)	CMS− *vs.* controls	CMS+ *vs.* controls	CMS+ *vs.* CMS−
Clinical						
Age (years)	56 ± 18	52 ± 12	56 ± 11	0.614 (between groups)		
Hb (g/dL)	15.0 ± 1.3	17.2 ± 1.1*	20.9 ± 1.7*†	<0.001	<0.001	<0.001
Hct (%)	46 ± 4	52 ± 4*	63 ± 6*†	0.002	<0.001	<0.001
S_{aO_2} (%)	97 ± 0	91 ± 5*	88 ± 4*†	<0.001	<0.001	0.044
caO_2 (mg/dL)	20.2 ± 1.8	21.6 ± 1.8	25.5 ± 2.3	0.147	<0.001	<0.001
CMS score (points)	0 ± 0	2 ± 2	8 ± 5*†	0.369	<0.001	<0.001
Anthropometrics						
Body mass (kg)	82.0 ± 13.4	71.6 ± 8.9	78.2 ± 11.8	0.053 (between groups)		
Stature (m)	1.77 ± 0.06	1.63 ± 0.04	1.62 ± 0.06	<0.001	<0.001	1.000
BMI (units)	26 ± 4	27 ± 4	30 ± 4*	1.000	0.025	0.122
Waist:hip	0.93 ± 0.08	0.95 ± 0.05	0.99 ± 0.04*	1.000	0.019	0.162
Education						
Secondary (*n*/%)	16/94	13/93	20/87	0.697 (between groups)		
University (*n*/%)	8/47	4/29	5/22	0.225 (between groups)		

Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; Hb, haemoglobin; Hct, haematocrit; S_{aO_2}, arterial oxyhaemoglobin saturation; caO_2, arterial oxygen content; BMI, body mass index. *Different *vs.* lowlanders ($P < 0.05$); †different *vs.* CMS− ($P < 0.05$). Clinical and anthropometric data were analysed using one-way ANOVAs and *post hoc* Bonferonni-adjusted independent samples *t*-tests. Education data were analysed using Pearson chi-square tests.

Free radicals. The ascorbate free radical ($A^{\bullet -}$) was used as a direct measure of systemic free radical formation (Buettner & Jurkiewicz, 1993). We injected 1 mL of plasma into a high-sensitivity multiple-bore sample cell (AquaX, Bruker Daltonics Inc., Billerica, MA, USA) housed within a TM_{110} cavity of an electron paramagnetic resonance spectrometer operating at X-band (9.87 GHz). We recorded samples by cumulative signal averaging of 10 scans using the following instrument parameters: resolution, 1024 points; microwave power, 20 mW; modulation amplitude, 0.65 G; receiver gain, 2×10^5; time constant, 40.96 ms; sweep rate, 0.14 G/s; sweep width, 6 G; centre field, 3486 G. We filtered spectra identically (moving average, 15 conversion points) using WINEPR software (Version 2.11, Bruker, Karlsruhe, Germany) and determined the double integral of each doublet using specialist software (OriginLab Corps, Northampton, MA, USA). The intra- and inter-assay CVs were both <5%.

Inflammatory stress.

Myeloperoxidase (MPO) activity. We employed a high-throughput, sensitive and homogeneous fluorescence-based method for detection of MPO chlorination activity using 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde oxime as a selective probe for hypochlorous acid as recently described (Stocker *et al.* 2017). The intra- and inter-assay CVs were both <5%.

Nitrosative stress. We measured plasma NO metabolites using ozone-based chemiluminescence (OBC) as outlined below (Bailey *et al.* 2017*a*).

S-Nitrosothiols (RSNO). Plasma (400 μL) was mixed with 5% acidified sulphanilamide and left to incubate in the dark at 21°C for 15 min to remove NO_2^- before injection into tri-iodide reagent for direct measurement of RSNO.

Nitrite (NO_2^-). A separate sample (200 μL) was also injected into tri-iodide reagent for the combined measurement of NO_2^- and RSNO with NO_2^- calculated by subtracting the concentration of RSNO. We performed all calculations using Origin/Peak Analysis software. The intra- and inter-assay CVs were 7% and 10%, respectively.

Total bioactive NO. This was calculated as the sum of RSNO + NO_2^-.

Haemodynamic assessments. We performed all resting measurements following 10 min of seated rest breathing room air at the prevailing barometric pressures in all groups (normoxic normocapnia for lowlanders, hypoxic hypocapnia for highlanders). Measurements were also repeated following the administration of hyperoxia ($F_{IO_2} = 1.0$, ~10 L/min for 10 min) to the inspired air in the highlanders only (hyperoxic normocapnia).

Cardiopulmonary function. We used finger photoplethysmography (Finometer PRO, Finapres Medical Systems, Amsterdam, the Netherlands) to monitor beat-to-beat mean arterial pressure (MAP) using the Model Flow method that incorporates participant sex, age, stature and mass (BeatScope 1.0 software; TNO; TPD Biomedical Instruments) to calculate stroke volume (SV) and cardiac output ($\dot{Q}$). We corrected for vertical displacement of the finger cuff relative to heart level using a reference probe placed on the chest at the fourth intercostal space in the mid-clavicular line. We measured heart rate (HR) using a lead II electrocardiogram (Dual BioAmp; ADInstruments, Oxford, UK). We sampled end-tidal partial pressures of oxygen and carbon dioxide ($PET_{O2/CO2}$) from a leak-free mask and analysed via capnography (ML 206, ADInstruments Ltd, Oxford, UK). Pulse oximetry (Nonin 9550 Onyx II, Nonin Medical, Inc., Plymouth, MI, USA) monitored arterial oxyhaemoglobin saturation (S_{aO_2}) on the third digit of the right hand.

Cerebrovascular function.

Cerebral blood flow. We insonated the M1 segment of the right middle cerebral artery (MCA) at a depth of 40–60 mm using a 2 MHz pulsed trans-cranial Doppler (TCD) ultrasound system (Multi-Dop X4, DWL Elektroniche Systeme GmbH, Sipplingen, Germany) to yield MCA velocity (MCAv). A headband device (Spencer Technologies, Nicolet Instruments, Madison, WI, USA) secured the Doppler probe over the trans-temporal window to achieve optimal insonation position and was maintained in this position for the duration of the study to avoid movement artefacts. We calculated cerebrovascular and total peripheral resistance (CVR and TPR) as MAP/MCAv or $\dot{Q}$ and cerebrovascular conductance index (CVCi) as MCAv/MAP. We calculated pulsatility index (PI) as systolic MCAv – diastolic MCAv/MCAv and further normalised the PI relative to the prevailing MAP. We calculated cerebral O_2 delivery (CDO_2) as the product of (arterial) O_2 content [$c(a)O_2$] ($1.39 \times Hb \times S_{aO_2}/100$) and MCAv. In order to normalise for the cerebral vasoconstrictor effects of polycythaemia and hypocapnia in the highlanders, we adjusted absolute CBF values for differences in (elevated) Hct and (lower) PET_{CO2} using the following equations:

$$\textit{Polycythaemia:}\ CBF_{/Hct} = 90 \times CBF(\text{measured})/(135 - Hct)$$

(Severinghaus, 2001)

$$\textit{Hypocapnia:}\ CBF_{/PETCO2} = CBF(\text{measured})/[1 + (PET_{CO2HYPOXIA} - PET_{CO2NORMOXIA^*}) \times 0.03]$$

(Bailey *et al.* 2009*b*) where the asterisk indicates fixed PET_{CO2} of 40 mmHg.

Data sampling. We sampled beat-by-beat data continuously at 1 kHz using an analog-to-digital converter (Powerlab/16SP ML795; ADInstruments) stored on a personal computer for off-line analysis (Chart version 7.2.2, ADInstruments). We gave chart files a coded number (not named) by an investigator blinded to the study. We 'time-aligned' the MAP and TCD channels given the time delay (1.07 s) associated with MAP signal processing when using the Finometer PRO device.

Cerebrovascular reactivity to CO_2 (CVR_{CO2}). Following 10 min of breathing room air, the inspirate was rapidly changed to 5% CO_2 with 21% O_2 and balanced nitrogen for 5 min at the prevailing barometric pressure. Following 5 min of recovery breathing room air, participants hyperventilated at 15 breaths/min for 5 min. From this, we calculated CVR_{CO2} as the percentage increase/decrease in MCAv from baseline per 1 mmHg increase/decrease in PET_{CO2} recorded during the final 30 s (average taken) of the hypercapneic/hypocapneic challenge having achieved steady-state:

$$CVR_{CO2}(\%) = 100 \times \frac{MCA_v(final) - MCA_v(baseline)}{MCA_v(baseline)} \Big/ [PET_{CO2}(final) - PET_{CO2}(baseline)]$$

(Bailey *et al.* 2013*a*).

From these data, we derived the CVR_{CO2} range as a useful indication of the cerebral circulation's combined ability to respond to differential changes in CO_2. We calculated the CVR_{CO2} range as the sum of the fractional vasodilatation and vasoconstriction incurred during the respective hypercapnoea and hypocapnia challenges as described:

$$CVR_{CO2}\text{range}(\%) = CVR_{CO2[\text{Hypercapnia}(\%)]} + CVR_{CO2[\text{Hypercapnia}(\%)]}$$

(Bailey *et al.* 2013*a*).

Dynamic cerebral autoregulatory capacity (dCA). We used a combination of spontaneous (seated) and driven (repeated squat-stands) oscillations in blood pressure (BP) and MCAv to assess dCA via transfer function analysis (TFA). Following 10 min of rest in the seated position, we obtained a 5 min segment of BP and MCAv data for spectral analysis of spontaneous oscillations. To increase BP variability and improve the reliability and interpretation of the TFA metrics (Katsogridakis *et al.* 2013), participants then performed 5 min periods of repeated squat-stand manoeuvres at randomly assigned frequencies of 0.05 Hz [10 s squat, 10 s standing) and 0.10 Hz (5 s squat, 5 s standing) with 5 min of standing rest between

frequencies (Smirl *et al.* 2015). During these manoeuvres, we instructed participants to maintain normal breathing and to avoid Valsalva. Beat-to-beat MAP and MCAv signals were calculated across each cardiac cycle, linearly interpolated and resampled at 2 Hz for TFA (Zhang *et al.* 1998) in accordance with the recommendations of the Cerebral Autoregulation Research Network (Claassen *et al.* 2016). Spontaneous MAP and MCAv power spectrum density and the mean value of TFA coherence gain, and phase of the spontaneous oscillations were band averaged across the very-low-frequency (VLF: 0.02–0.07 Hz, 50 to 14.3-second cycles) and low-frequency (LF: 0.07–0.20 Hz, 14.3 to 5 s cycles) ranges where CA is most operant (Zhang *et al.* 1998). The TFA coherence, gain and phase of the driven MAP oscillations were sampled at the driven frequencies (0.05 or 0.10 Hz). To ensure we entered robust phase and gain estimates for analysis, we averaged only those gain and phase (positive to eliminate wrap-around) values where the corresponding coherence was $\geq$0.5. Accordingly, we interpreted an increase in gain and decrease in phase to reflect impaired dCA indicative of a more pressure-passive relationship between MAP and MCAv.

Cognitive function

Psychometric tests. We conducted a battery of psychometric tests with three consecutive testing periods to ensure habituation and to avoid the confounding influence of learning effects as previously outlined (Marley *et al.* 2017):

(1) Learning and memory. Rey Auditory Verbal Learning consists of two lists containing 15 unrelated words. We read List 1 aloud at a rate of 1 word per second. The participant recalled as many of the 15 words in any order. We repeated this four times (total of five recalls, A1–A5). We read List 2 aloud and the participant recalled as many of the 15 words as possible, again in any order (B1). Finally, we asked the participant to recall as many words as possible from List 1 (delayed recall, A6) (Rey, 1958).

(2) Working memory. Repetition of Digits Backwards (RDB) is based on the Repetition of Digits test (Wechsler, 1955) which comprises digits forwards (RDF) and RDB. Both tests consist of seven pairs of random number sequences that the researcher reads aloud at a rate of one per second. RDF required the participant to recall the numbers in the correct sequence until they got the sequence wrong. The sequence began with three numbers and increased by one number every other pair. RDB required the participant to recall the numbers in the reverse order from the sequences they were presented, until they got the sequence wrong. The sequence began with two numbers and increased by one every other pair. We scored the tests by the total number of sequences re-called. Trail Making Test B (TMT-B) is based on the Army Individual Test Battery (War Department Adjutant General's Office, 1944) that consists of two parts, A (TMT-A) and TMT-B constructed with 25 circles distributed over a sheet of A4 paper. In part A, the circles are numbered 1–25; the participant connected the numbers in ascending order. In part B, the circles include both numbers (1–13) and letters (A–L); again participants connected the circles in ascending pattern, but alternating between number and letter (i.e. 1-A-2-B-3-C, etc.). We asked the participant to complete the task as quickly as possible without removing the pen from the paper until completion. We scored the tests as the time taken in seconds to complete each trail.

(3) Attention and information processing. We assessed RDF and TMT-A as outlined above. The Digit Symbol Substitution Test (DSST) consists of matching nine digits with their corresponding symbols. We gave the participant a piece of A4 paper with nine digits and their corresponding symbol and a grid of 93 digits; under each digit the participant drew as many of the corresponding symbols as possible within 90 s. We scored the test by how many they drew correctly within the alloted time (Wechsler, 1955). The Stroop test (ST) consists of two parts A and B, with 24 words written in different colours on a 4 $\times$ 6 grid. Test A required the participants to read aloud the word written as quickly as possible. Test B required the participant to read aloud the colour of the word written and not what the word said as quickly as possible (Stroop, 1935). This test was assessed in the highlander subgroups only and not the lowlanders due to logistical constraints.

(4) Visuo-motor coordination. The grooved pegboard dexterity test (GPDT) consists of two parts and required the participant to place 25 pegs in a pegboard containing equally sized holes with randomly positioned slots. Participants placed identical pegs with a key on one side that had to be rotated to match the hole before they could be inserted, into the 25 holes on the pegboard working from left to right, front to back. The test consisted of two parts, the first completed using the dominant hand (GPDT-D) and then repeated using the non-dominant hand (GPDT-ND). The test was scored by the time taken to complete in seconds (Klove, 1963).

Higher scores in the RAVLT-A/B and RDB/RDF tests and lower scores in the TMT-A/B, GPDT-D/GPDT-ND and Stroop A/B tests indicated superior performance.

Questionnaires. The original English questionnaires outlined below were used for the lowlanders whereas the equivalent validated Spanish versions were used for the highlanders.

Montreal Cognitive Assessment (MoCA). The MoCA measures visuospatial/executive, naming, memory,

attention, language, abstraction, delayed recall and orientation, with each section having a designated point value with a maximum score of 30 points (Nasreddine *et al.* 2005). We scored the test as the number of questions answered correctly and participants with 12 years or less of education had a point added to their total score. We graded MoCA on three levels: normal, 25.2–29.6 points; mild cognitive impairment, 19–25.2 points; and Alzheimer's disease, 11.4–21 points according to established guidelines (Nasreddine *et al.* 2005). Because MoCA assesses executive function, it is particularly useful for patients with vascular impairment, including vascular dementia (Sheehan, 2012).

Depression. We assessed depressive symptoms using the Becks Depression Inventory (BDI), which has been shown to be a reliable and valid measure of self-reported depression in both normal and psychiatric populations (Beck *et al.* 1961). The BDI is a self-report inventory with 21 items assessing the behavioural and cognitive symptoms of depression. Each item consists of four statements numbered from 0 to 3 with higher numbers indicating more severe depressive symptoms. We scored the questionnaire by the sum of all the responses with a maximum score of 63 points. A score of 0–9 points indicated minimal depression whereas scores of 10–18 points, 19–29 points and 30–63 points indicated mild, moderate and severe depression, respectively.

Statistical analysis

Power calculation. We analysed these data using G* Power 3.1 software. Assuming comparable differences and corresponding effect sizes previously observed in plasma $A^{\bullet-}$ ($\eta^2 = 0.54$) and NO_2^- ($\eta^2 = 0.67$) (Bailey *et al.* 2013*c*), our primary end-outcome variables for OXNOX stress, the present study required a (minimum) sample size of 24–36 participants (8–12 per group) in order to achieve a power of 0.80 at $P < 0.05$. We chose to further inflate this during recruitment given the potential for incomplete data collection.

Inferential statistics. We analysed these data using using the Statistics Package for Social Scientists (IBM SPSS Statistics Version 24.0). Shapiro-Wilk W tests ($P > 0.05$) confirmed that all data sets were normally distributed. We analysed demographic (Table 1), metabolic (Table 2), haemodynamic (Table 3), cognition and depression (Table 5), CVR_{CO2} (Fig. 2) and TFA (Fig. 3) datasets using one-way ANOVAs and *post hoc* Bonferonni-adjusted independent samples *t*-tests if we observed a main effect. We analysed education data (Table 1) using Pearson chi-square tests. We analysed responses to hyperoxia in the highlanders (Table 4) using two-way (subgroup × inspirate) ANOVAs and *post hoc* Bonferonni-adjusted paired samples (within subgroups) or independent (between subgroups) samples *t*-tests if we observed an interaction effect. We determined relationships between metabolic, haemodynamic and clinical variables (Fig. 4) using Pearson product moment correlations. We established significance at $P < 0.05$ for all two-tailed tests (with individual P values for all comparisons shown) and data are expressed as mean ± SD.

Results

Anthropometric data. All groups were of a comparable age and body mass whereas the highlanders were generally shorter with a corresponding elevation in body mass index (BMI) in CMS+ (Table 1).

Metabolic data.

Oxygenation and erythrocytosis. As anticipated, highlanders presented with arterial hypoxaemia with the most pronounced erythrocytosis observed in CMS+ (Table 1).

Oxidative stress. We observed lower GSH:GSSG in CMS+ due to the combination of lower GSH and higher GSSG (Table 2). We observed a reciprocal elevation in $A^{\bullet-}$ that was generally more pronounced in highlanders and further exaggerated in CMS+ (Table 2). Figure 3*A–C* provides typical examples of electron paramagnetic resonance (EPR) doublets observed exhibiting hydrogen hyperfine coupling constants (a^H) of ∼1.8 G ($g = 2.0052$).

Inflammatory stress. MPO activity was higher in CMS+ (Table 2).

Nitrosative stress. Plasma NO_2^- and RSNO were consistently lower in highlanders (Table 2) but not different between CMS+ and CMS−. Figure 3*D–F* provides typical examples of OBC traces observed.

Haemodynamic data

Cardiopulmonary. As anticipated, highlanders were more hypocapnic whereas we observed no between-group/subgroup differences in MAP, SBP, DBP, $\dot{Q}$, HR, SV or TPR (Table 3). Hyperoxia and the corresponding normalisation of S_{aO_2} decreased $\dot{Q}$ in the highlanders due to the combined decrease in both HR and SV and as a consequence was associated with elevated TPR (Table 4).

Cerebrovascular. Cerebral perfusion was generally lower in highlanders and was associated with a corresponding

Table 2. Metabolic data

Group:	Lowlanders	Highlanders		*P* values		
Subgroup:	Controls ($n = 17$)	CMS− ($n = 14$)	CMS+ ($n = 23$)	CMS− *vs.* controls	CMS+ *vs.* controls	CMS+ *vs.* CMS−
Oxidative stress						
GSH (μM)	N/A	549 ± 154	412 ± 151†	N/A	N/A	0.012
GSSG (μM)	N/A	176 ± 48	197 ± 34†	N/A	N/A	0.018
GSH:GSSG (AU)	N/A	3.3 ± 1.1	2.1 ± 0.9†	N/A	N/A	0.049
$A^{\bullet-}$ (AU)	29,450 ± 6,929	54,451 ± 20,722*	59,729 ± 18,133*†	<0.001	<0.001	0.042
Inflammatory stress						
MPO (μg/L)	N/A	609 ± 12	894 ± 168†	N/A	N/A	0.016
Nitrosative stress						
NO_2^- (nM)	249.1 ± 65.1	139.9 ± 76.7*	130.7 ± 83.6*	0.001	<0.001	1.000
RSNO (nM)	5.4 ± 3.0	5.0 ± 2.7	4.7 ± 3.9*	0.850 (between groups)		
Total bioactive NO (nM)	254.5 ± 64.2	144.9 ± 78.0*	135.4 ± 83.7*	0.001	<0.001	1.000

Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; GSH/GSSH, reduced/oxidised glutathione; $A^{\bullet-}$, ascorbate radical; NO, nitric oxide; NO_2^-, nitrite; RSNO, *S*-nitrosothiols; total bioactive NO (NO_2^- + RSNO); MPO, myeloperoxidase; N/A, not assessed. *Different *vs*. lowlanders ($P < 0.05$); †different *vs*. CMS− ($P < 0.05$). Data were analysed using independent samples *t*-tests (where lowlander data were unavailable) and one-way ANOVAs with *post hoc* Bonferonni-adjusted independent samples *t*-tests.

Table 3. Haemodynamic data

Group:	Lowlanders	Highlanders		*P* values		
Subgroup:	Controls ($n = 17$)	CMS− ($n = 14$)	CMS+ ($n = 23$)	CMS− *vs.* controls	CMS+ *vs.* controls	CMS+ *vs.* CMS−
MAP (mmHg)	79 ± 21	80 ± 10	87 ± 14	0.238 (between groups)		
SBP (mmHg)	118 ± 30	118 ± 18	128 ± 22	0.309 (between groups)		
DBP (mmHg)	61 ± 18	61 ± 8	67 ± 11	0.228 (between groups)		
$\dot{Q}$ (L/min)	7.42 ± 1.33	6.99 ± 0.80	7.24 ± 1.38	0.627 (between groups)		
HR (beats/min)	71 ± 14	71 ± 12	72 ± 14	0.990 (between groups)		
SV (mL)	105 ± 13	101 ± 22	103 ± 22	0.853 (between groups)		
TPR (mmHg/L/min)	10.97 ± 2.93	11.60 ± 1.73	12.41 ± 2.80	0.231 (between groups)		
S_{aO_2} (%)	97 ± 0	91 ± 5*	88 ± 4*†	<0.001	<0.001	0.044
PET_{O2} (mmHg)	107 ± 5	54 ± 3*	51 ± 5*	<0.001	<0.001	0.236
PET_{CO2} (mmHg)	39 ± 3	34 ± 5*	33 ± 4*	0.019	0.022	1.000
Cerebrovascular						
MCAv (cm/s)	49 ± 12	39 ± 12*	35 ± 12*†	0.044	0.001	0.048
$MCAv_{/Hct}$ (cm/s)	50 ± 11	42 ± 13*	43 ± 14*	0.039	0.037	1.000
$MCAv_{/PETCO2}$ (cm/s)	51 ± 11	48 ± 16	44 ± 15*	1.000	0.040	1.000
PI (AU)	1.04 ± 0.18	1.15 ± 0.41	1.45 ± 0.39*†	1.000	0.002	0.048
Normalised PI (AU)	0.014 ± 0.006	0.014 ± 0.005	0.017 ± 0.004	0.216 (between groups)		
CVR (mmHg/cm/s)	1.74 ± 0.69	2.27 ± 0.80	2.89 ± 0.57*†	0.667	0.010	0.037
CVCi (cm/s/mmHg)	0.70 ± 0.39	0.49 ± 0.16	0.42 ± 0.18*†	0.088	0.003	0.010
CDO_2 (mL/cm/s)	983 ± 209	850 ± 305*	889 ± 303*	0.046	0.047	1.000

Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; $\dot{Q}$, cardiac output; SV, stroke volume; TPR, total peripheral resistance; S_{aO_2}, arterial oxyhaemoglobin saturation; $PET_{O2/CO2}$, end-tidal partial pressure of oxygen/carbon dioxide; MCAv, middle cerebral artery velocity; SMCAv/DMCAv, systolic/diastolic MCAv; $MCAv_{/Hct}$, MCAv (retrospectively) adjusted for differences in haematocrit (Hct); $MCAv_{/PETCO2}$, MCAv (retrospectively) adjusted for differences in end-tidal partial pressure of carbon dioxide, see Methods; AU, arbitrary units; CVR, cerebrovascular resistance; CVCi, cerebrovascular conductance index; CDO_2, cerebral oxygen delivery. *Different *vs*. lowlanders ($P < 0.05$); **different *vs*. hypoxia for given highlanders subgroup ($P < 0.05$); †different *vs*. CMS− for given inspirate ($P < 0.05$). Data were analysed using one-way ANOVAs and *post hoc* Bonferonni-adjusted independent samples *t*-tests.

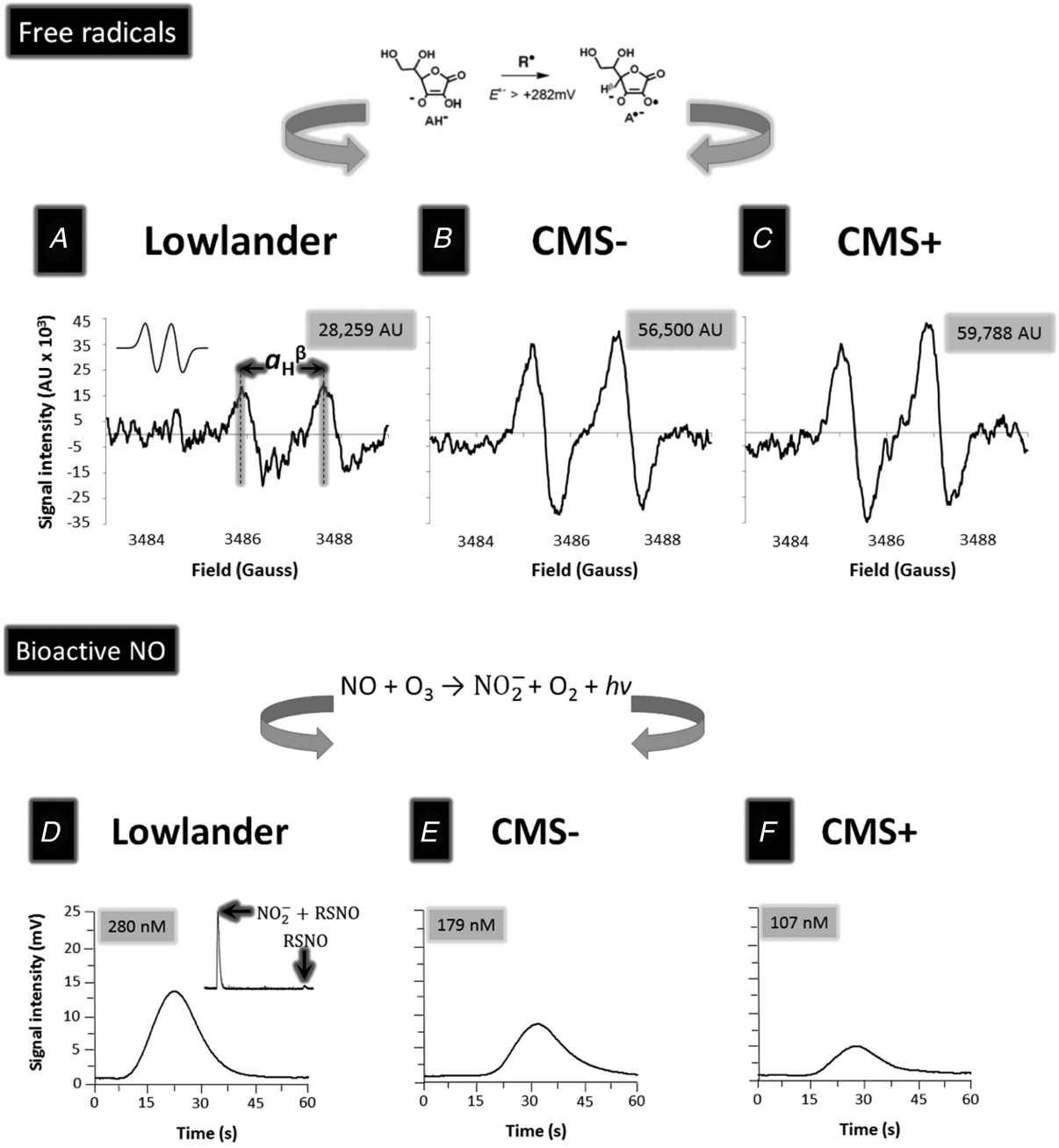

Figure 3. Typical electron paramagnetic resonance (EPR) spectra of the plasma ascorbate radical (A–C) and ozone-based chemiluminescence detection of bioactive (nitrite + *S*-nitrosothiols) nitric oxide metabolites (D–F) at rest in the systemic circulation of a lowlander and highlanders without (CMS–) and with (CMS+) chronic mountain sickness

A–C, oxidation of the ascorbate monoanion (AH^-) by any free radical ($R^\bullet$) with a one-electron reduction potential that exceeds +282 mV will yield $A^{\bullet-}$ (schematic illustrated above). The unpaired electron is delocalised over a highly conjugated tri-carbonyl π-system, rendering it resonance-stabilised and thereby facilitating direct detection by EPR spectroscopy. At the current settings, $A^{\bullet-}$ appears as a (filtered) doublet with a hydrogen hyperfine coupling constant ($a_H{}^\beta$) of ∼1.76 G (see inset to *A* for simulated spectrum). *D–F*, filtered traces of bioactive nitric oxide (NO) metabolites (nitrite + *S*-nitrosothiols) generated via ozone-based chemiluminescence involving the reaction of NO with ozone (O_3) that yields a photon ($h\nu$) and subsequent conversion to a potential difference. Insert top right highlights the composite signals (before and after sulphanilamide incubation) for separate measurement of nitrite (NO_2^-) and *S*-nitrosothiols (RSNO). Note general elevations in the signal intensity (AU, arbitrary units) of $A^{\bullet-}$ and reciprocal decrease in the circulating concentration of bioactive NO metabolites in the highlanders, especially in the patient with CMS. Spectra were chosen to best reflect the average signal intensities observed in each of the respective groups.

Table 4. Responses to hyperoxia in highlanders

Subgroup:	CMS− ($n = 14$)		CMS+ ($n = 23$)		*P* values		
Inspirate:	Hypoxia	Hyperoxia	Hypoxia	Hyperoxia	Subgroup	Inspirate	Interaction
Cardiopulmonary							
MAP (mmHg)	80 ± 10	83 ± 8	87 ± 14	87 ± 16	0.188	0.500	0.405
SBP (mmHg)	118 ± 18	121 ± 13	128 ± 22	125 ± 24	0.284	0.885	0.267
DBP (mmHg)	61 ± 8	64 ± 7	67 ± 11	68 ± 13	0.167	0.241	0.426
$\dot{Q}$ (L/min)	6.99 ± 0.80	6.11 ± 0.77	7.24 ± 1.38	6.17 ± 1.22	0.663	<0.001	0.540
HR (beats/min)	71 ± 12	68 ± 11	72 ± 14	67 ± 14	0.993	<0.001	0.456
SV (mL)	101 ± 22	92 ± 21	103 ± 22	95 ± 28	0.746	0.001	0.863
TPR (mmHg/L/min)	11.60 ± 1.73	13.81 ± 2.00	12.41 ± 2.80	14.55 ± 3.99	0.385	<0.001	0.948
S_{aO_2} (%)	91 ± 5	97 ± 1**	88 ± 4†	97 ± 1**	0.043	<0.001	0.043
PET_{O2} (mmHg)	54 ± 3	374 ± 81	51 ± 5	380 ± 51	0.887	<0.001	0.699
PET_{CO2} (mmHg)	34 ± 5	35 ± 6	33 ± 4	39 ± 4	0.007	0.385	0.847
Cerebrovascular							
MCAv (cm/s)	39 ± 12	39 ± 9	35 ± 12†	34 ± 7†	0.052	0.756	0.029
$MCAv_{/Hct}$ (cm/s)	42 ± 13	43 ± 11	43 ± 14	42 ± 8	0.939	0.759	0.626
$MCAv_{/PETCO2}$ (cm/s)	48 ± 16	48 ± 14	44 ± 15	36 ± 11**†	0.045	0.009	0.011
PI (AU)	1.15 ± 0.41	0.99 ± 0.18	1.45 ± 0.39†	1.21 ± 0.27**	0.013	0.001	0.041
Normalised PI (AU)	0.014 ± 0.005	0.012 ± 0.003	0.017 ± 0.004	0.015 ± 0.006	0.068	0.010	0.803
CVR (mmHg/cm/s)	2.27 ± 0.80	2.23 ± 0.53	2.89 ± 0.57	2.67 ± 0.74	0.054	0.518	0.640
CVCi (cm/s/mmHg)	0.49 ± 0.16	0.47 ± 0.11	0.42 ± 0.18	0.40 ± 0.12	0.119	0.504	0.861
CDO_2 (mL/cm/s)	850 ± 305	909 ± 243	889 ± 303	951 ± 193	0.626	0.083	0.981

Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; $\dot{Q}$, cardiac output; SV, stroke volume; TPR, total peripheral resistance; S_{aO_2}, arterial oxyhaemoglobin saturation; $PET_{O2/CO2}$, end-tidal partial pressure of oxygen/carbon dioxide; MCAv, middle cerebral artery velocity; SMCAv/DMCAv, systolic/diastolic MCAv; $MCAv_{/Hct}$, MCAv (retrospectively) adjusted for differences in haematocrit (Hct); $MCAv_{/PETCO2}$, MCAv (retrospectively) adjusted for differences in end-tidal partial pressure of carbon dioxide, see Methods; AU, arbitrary units; CVR, cerebrovascular resistance; CVCi, cerebrovascular conductance index; CDO_2, cerebral oxygen delivery. **Different *vs.* hypoxia for given subgroup ($P < 0.05$); †different *vs.* CMS− for given inspirate ($P < 0.05$). Data were analysed using two-way (subgroup × inspirate) ANOVAs and *post hoc* Bonferonni-adjusted paired (within subgroups) or independent (between subgroups) samples *t*-tests.

elevation in CVR and PI and reciprocal decrease in CVCi and CDO_2 that was most marked in CMS+ (Table 3). While hyperoxia failed to normalise perfusion, it tended to restore CDO_2 subsequent to an elevation in caO_2 (Table 4). The lower cerebral perfusion still persisted in CMS+ when CBF was (retrospectively) adjusted for the independent vasoconstrictor effect of hypocapnoea but not polycythaemia (Table 4). Highlanders exhibited a lower CVR_{CO2} range due primarily to a blunted response to hypercapnoea (Fig. 4) whereas we observed no differences between CMS+ and CMS−. Figure 5 illustrates typical waveform data for a representative patient with CMS+ and corresponding spectral analysis for both spontaneous (resting) and driven (squat-stand manoeuvres) oscillations in MAP and MCAv. Spontaneous and driven TFA metrics are illustrated in Fig. 6. During spontaneous TFA with moderate coherence values (Fig. 6*A*), LF phase was higher in CMS+ (Fig. 6*B*) with no (between-group) differences observed in gain at either frequency (Fig. 6*C*). The driven protocols, although not altering PET_{CO2} ($P < 0.05$ *vs.* spontaneous, data not shown), resulted in markedly amplified oscillations culminating in an augmented signal-to-noise ratio as confirmed by the 106–238-fold (0.05 Hz) and 665–1320-fold (0.10 Hz) increase in BP spectral power and 133–374-fold (0.05 Hz) and 424–1039-fold (0.10 Hz), increase in MCAv spectral power (compared to spontaneous VLF/LF measures at the respective frequency of interest) resulting in TFA coherence values exceeding 0.95 AU in all cases (Fig. 6*D*). Despite no differences in phase (Fig. 6*E*), we observed consistently lower (35–40%) point estimates of VLF and LF gain in the highlanders with no differences observed between CMS+ and CMS− (Fig. 6*F*).

Clinical data

Cognitive function. We observed consistently lower performances on RAVLT, RDB, RDF, TMT-B and DSST in the highlanders, with the lowest performance recorded for RAVLT-A6 and Stroop Task-A in CMS+ (Table 5). We observed comparable MoCA scores between CMS− and

lowlanders and consistently lower scores in CMS+, with 87% of the group (20/23 participants) scoring ≤25 points (Table 5).

Depression. Likewise, we observed comparable BDI scores between CMS− and lowlanders and consistently lower scores in CMS+ (Table 5).

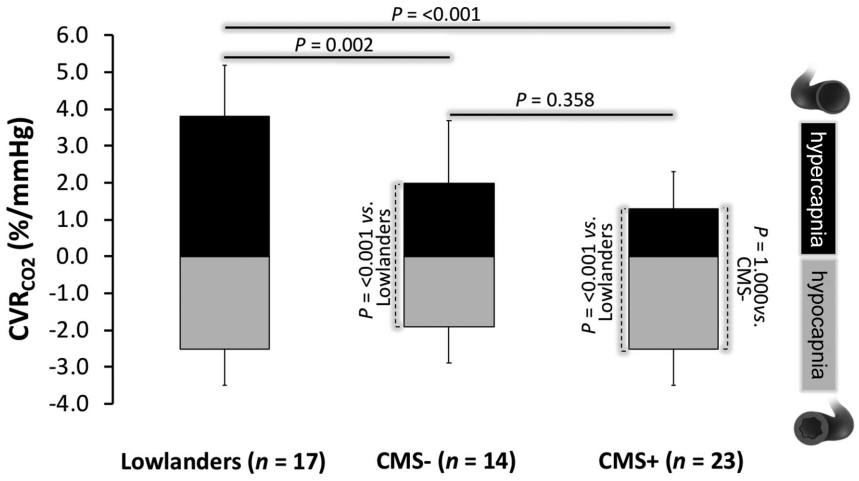

Figure 4. Cerebrovascular reactivity to carbon dioxide (CVR_{CO2})
Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; *different *vs*. lowlanders for respective challenge ($P < 0.05$). Individual hypocapnia and hypercapnia datasets were analysed using one-way ANOVAs and *post hoc* Bonferonni-adjusted independent samples *t*-tests.

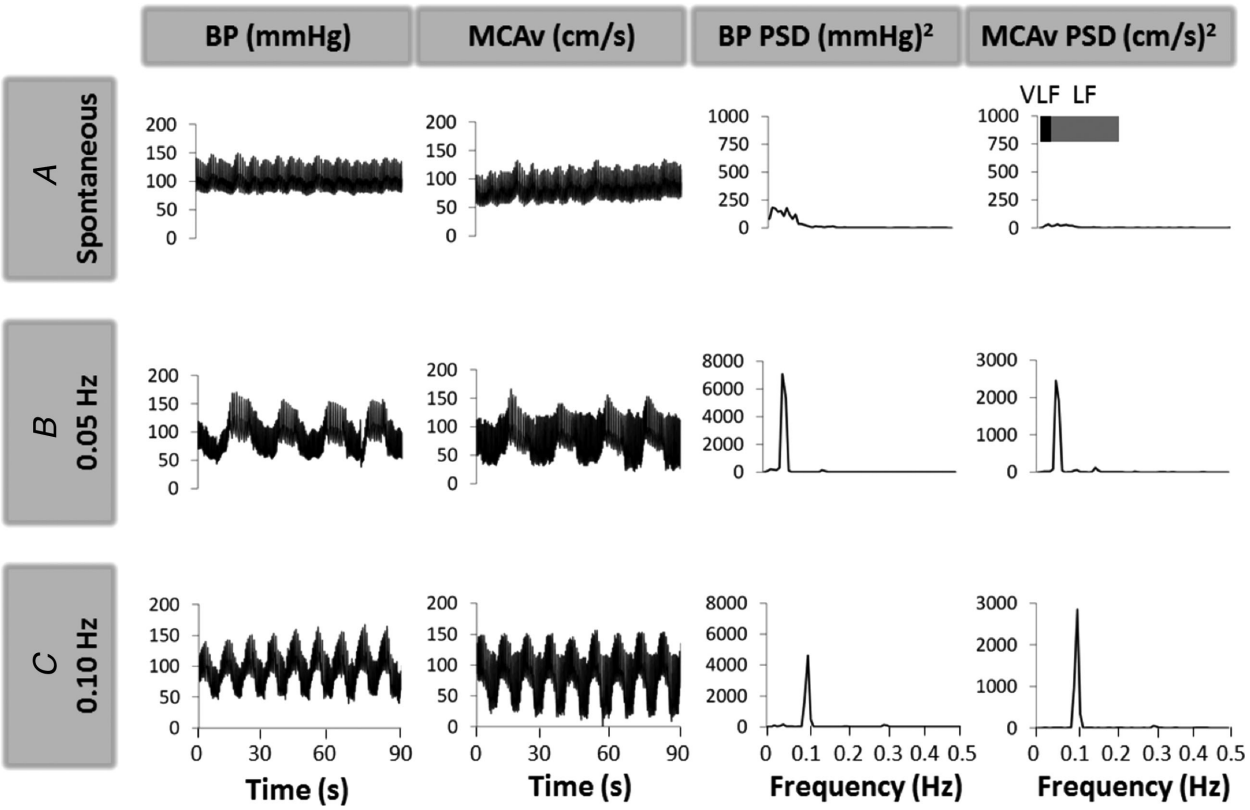

Figure 5. Typical waveforms and spectral analysis of haemodynamic responses observed during spontaneous (*A*) and repeated squat-stand manoeuvres (*B*, *C*) in a representative patient with chronic mountain sickness
BP, blood pressure; MCAv, middle cerebral artery velocity; PSD, power spectral density; V/LF, very/low frequency. Note the markedly amplified and coherent oscillations in MAP and MCAv during the repeated squat-stand manoeuvres compared to resting (spontaneous) measures, leading to improved estimation of transfer function of dynamic cerebral autoregulation at the frequency of interest.

Correlations

We observed inverse relationships between $A^{\bullet-}$ and the following variables: S_{aO_2}, total bioactive NO, MCAv and $CVR_{CO2Hyper}$ (Fig. 7*A–D*). We observed positive relationships between total bioactive NO and the following variables: CDO_2 and MoCA (Fig. 7*E*, *F*) and some aspects of cognitive function, namely performance on the RAVLT-A1-A5 ($r = 0.438$, $P = 0.001$), RAVLT-B1 ($r = 0.358$, $P = 0.008$), RAVLT-A6 ($r = 0.270$, $P = 0.048$), RDF ($r = 0.536$, $P < 0.001$), RDB ($r = 0.565$, $P < 0.001$) and DSST ($r = 0.266$, $P = 0.052$).

Discussion

More than 150 million HA dwellers are permanently exposed to hypoxaemia, a problem predisposing lowlanders suffering from cardiopulmonary disease to cognitive dysfunction and dementia. Here, we show for the first time that systemic OXINOS was permanently elevated in healthy well-adapted (CMS−) highlanders and accompanied by a proportional decrease in cerebral perfusion and blunted reactivity to hypercapnia. These alterations were associated with a mild decrease in cognitive performance, with learning/memory and attention/information processing the domains being most affected, but without any clinical evidence for depression. Second and in stark contrast, the sustained elevation in systemic OXINOS was further exaggerated in maladapted (CMS+) highlanders and accompanied by more pronounced impairments in cerebrovascular function, cognition and clinical symptoms of depression. Collectively, these findings are the first to suggest that a physiological continuum may exist for hypoxaemia-induced systemic OXINOS that when excessive is associated with accelerated cognitive decline and depression, helping identify those in need of more specialist neurological follow-up and targeted support.

Metabolic function

Because the concentration of ascorbate in human plasma is orders of magnitude greater than any oxidising free radical combined with the low one-electron reduction potential for the $A^{\bullet-}$/ascorbate monanion (AH^-) couple ($E^o = 282$ mV) (Williams & Yandell, 1982), any oxidising species ($R^{\bullet}$) generated within the systemic circulation will result in the one-electron oxidation of ascorbate to form the distinctive EPR-detectable $A^{\bullet-}$ doublet ($AH^- + R^{\bullet} \rightarrow A^{\bullet-} + R\text{-}H$, Fig. 3*A–C*) (Buettner, 1993). Thus, the elevations observed in plasma $A^{\bullet-}$ provide direct evidence that systemic free radical formation was permanently elevated in the highlanders, approximately double that observed in the lowlanders, with the highest concentrations recorded in the comparatively more hypoxaemic CMS+ patients.

These observations combined with the inverse relationships consistently observed between $A^{\bullet-}$ and S_{aO_2}

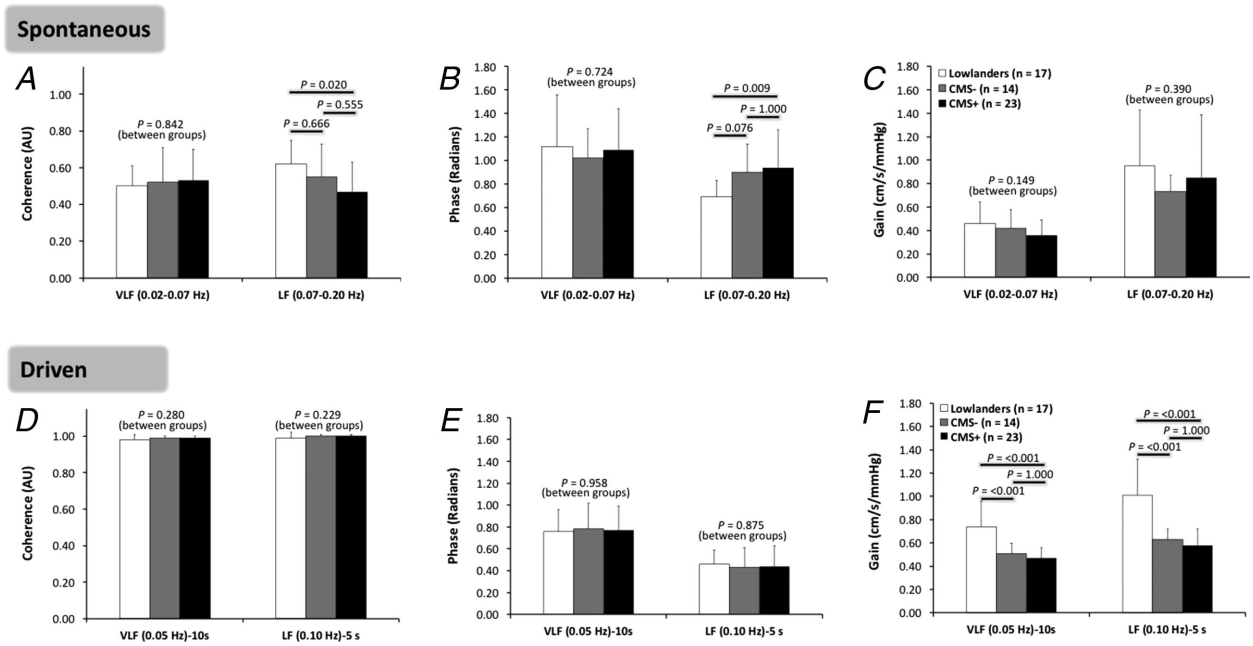

Figure 6. Transfer function analysis of the cerebral pressure–flow relationship during spontaneous (*A–C*) and driven (*D–F*) oscillations in blood pressure and middle cerebral artery velocity
Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; *different *vs.* lowlanders for the given frequency ($P < 0.05$). Individual frequency data sets were analysed using using one-way ANOVAs and *post hoc* Bonferonni-adjusted independent samples *t*-tests.

Table 5. Cognition and depression

Group:	Lowlanders	Highlanders		*P* values		
Subgroup:	Controls ($n = 17$)	CMS− ($n = 14$)	CMS+ ($n = 23$)	CMS− *vs.* controls	CMS+ *vs.* controls	CMS+ *vs.* CMS−
Learning and memory						
Rey Auditory Verbal Learning Test A1–A5 (*n*)	48 ± 8	34 ± 9*	30 ± 9*	<0.001	<0.001	0.538
Rey Auditory Verbal Learning Test B1 (*n*)	5 ± 1	4 ± 1	3 ± 1*	0.335	<0.001	0.116
Rey Auditory Verbal Learning Test A6 (*n*)	9 ± 4	8 ± 3	5 ± 2*†	0.392	<0.001	0.044
Working memory						
Repetition of Digits Backwards (*n*)	6 ± 2	4 ± 1*	4 ± 2*	<0.001	<0.001	1.000
Trail Making Test B (s)	74 ± 31	106 ± 43	109 ± 51*	0.137	0.044	1.000
Attention/information processing						
Repetition of Digits Forwards (*n*)	8 ± 2	4 ± 2*	4 ± 2*	<0.001	<0.001	1.000
Trail Making Test A (s)	37 ± 15	41 ± 18	48 ± 17	0.155 (between groups)		
Digit Symbol Substitution Test (*n*)	54 ± 12	42 ± 12*	37 ± 13*	0.034	<0.001	0.555
Stroop Task A (s)	No data	14 ± 2	17 ± 5†	N/A	N/A	0.048
Stroop Task B (s)	No data	38 ± 14	41 ± 15	N/A	N/A	0.555
Montreal Cognitive Assessment (points)	26 ± 3	24 ± 4	21 ± 5*†	0.676	0.001	0.044
Montreal Cognitive Assessment Score ≤25 points (*n*/%)	5/29	7/50	20/87*†	0.241	<0.001	0.014
Visuomotor coordination						
Grooved Pegboard Dexterity Test-Dominant (s)	73 ± 11	67 ± 8	71 ± 12	0.296 (between groups)		
Grooved Pegboard Dexterity Test-Non Dominant (s)	83 ± 24	72 ± 11	81 ± 23	0.309 (between groups)		
Depression						
Beck's Depression Inventory (points)	6 ± 5	7 ± 10	16 ± 13*†	1.000	0.014	0.044

Values are mean ± SD; CMS−/CMS+, highlanders without/with chronic mountain sickness; *n*, number correct. *Different *vs.* lowlanders ($P < 0.05$); †different *vs.* CMS− ($P < 0.05$). Data were analysed using one-way ANOVAs and *post hoc* Bonferonni-adjusted independent samples *t*-tests with the exception of MoCA cut-point (≤25 points) analysed using Pearson chi-square tests with standardised residuals.

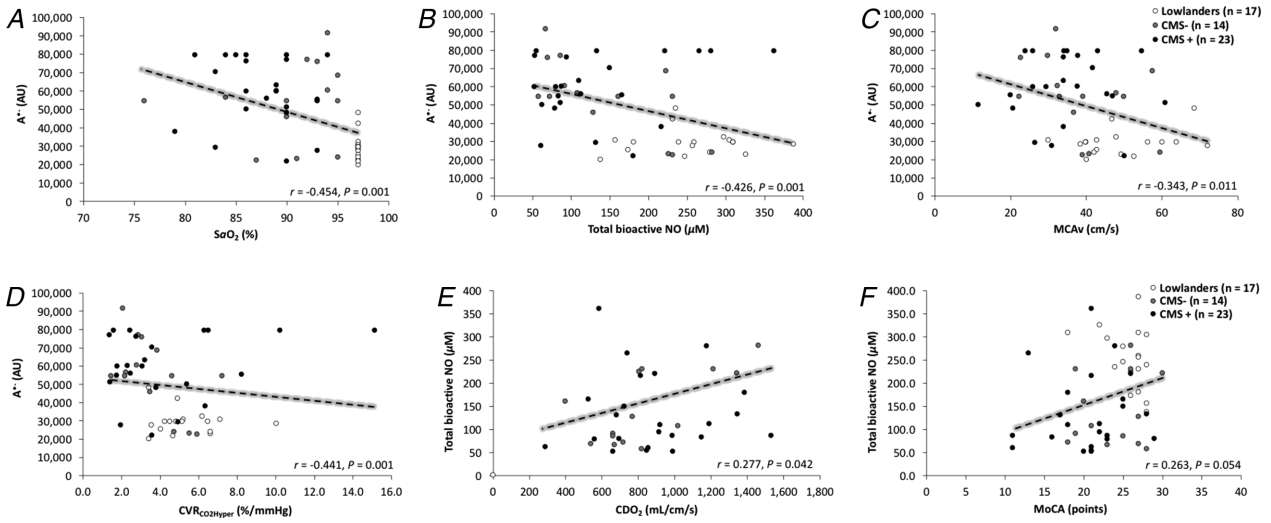

Figure 7. Relationships between metabolic, haemodynamic and clinical correlates in lowlanders and highlanders with and without chronic mountain sickness (CMS)
A, ascorbate radical; S_{aO_2}, arterial oxyhaemoglobin saturation; NO, nitric oxide; MCAv, middle cerebral artery blood flow velocity; CDO_2, cerebral oxygen delivery; $CVR_{CO2Hyper}$, cerebrovascular reactivity to carbon dioxide in the hypercapnic range; MoCA, Montreal Cognitive Assessment. Data were analysed using a Pearson product moment correlation.

suggest that hypoxaemia may have been the upstream stimulus for oxidative catalysis, thus confirming our original hypothesis proposed in Fig. 1. Our findings agree with an evolving body of literature indicating that hypoxaemia catalyses not only systemic but also local cerebral free radical formation (Bailey *et al.* 2009*c*, 2018*a*) classically attributed to mitochondrial superoxide ($O_2^{\bullet-}$) release by complex III of the electron transport chain, notwithstanding additional contributions from other (i.e. extra-mitochondrial) sources (Waypa *et al.* 2010). Systemic free radical formation coincided with the selective oxidation of GSH, the most abundant intracellular thiol found in the brain, which probably reflects an attempt to constrain OXINOS through the targeted scavenging of reactive oxygen, nitrogen and carbon-centred species (Rae & Williams, 2017).

Oxidative stress coincided with both inflammatory and nitrosative stress as confirmed by the combined, sustained elevation in MPO and lower NO_2^- and RSNO, respectively, culminating in OXINOS that was most pronounced in CMS+. These findings extend our previous observations of a permanent and graded elevation in systemic OXNOS stress ranging from moderate in CMS− to severe in CMS+ (Bailey *et al.* 2013*c*). Because plasma NO_2^- is a source of NO with conversion catalysed by deoxyhaemoglobin-mediated reduction and acidic disproportionation (Gladwin *et al.* 2000; Cosby *et al.* 2003) we interpreted the more exaggerated decrease in CMS+ to be one of the contributory factors responsible for the observed impairment in systemic vascular function in the form of blunted flow-mediated dilatation and increased arterial stiffness (Bailey *et al.* 2013*c*). To further extend these observations, we sought to determine if the same concept applied more locally to the hypoxic cerebrovasculature, including to what extent exaggerated OXINOS associates with the neurological deficits underpinning CMS.

Haemodynamic function

Consistent with previous studies (Jansen & Basnyat, 2011), highlanders, in particular CMS+, exhibited ∼30% lower cerebral perfusion, notwithstanding the interpretive constraints associated with TCD ultrasound (Liu *et al.* 2017) and corresponding decrease in CDO_2 that persisted even following correction for the independent vasoconstrictor effects of polycythaemia-induced alteration in blood rheology and hypocapnia. The decrease in cerebral perfusion coincided with an impaired ability of the cerebrovasculature to respond to vasodilator stimuli, notably hyperoxia and hypercapnia, the latter being consistently more pronounced in CMS+. The consistent relationships observed between total bioactive NO, MCAv and $CVR_{CO2Hyper}$, although not disassociating cause from effect, support a potential contributory role for OXINOS and corresponding decrease in vascular NO availability, consistent with previous studies that have highlighted endothelial-derived NO as an important, albeit not exclusive, mediator of cerebral (hyper)perfusion (Lavi *et al.* 2003). Interestingly, OXINOS appeared to exert more of an effect on the cerebral than systemic circulation given the lack of difference in MAP and TPR in CMS+.

Furthermore, we have previously identified that NO plays an important role in the maintenance of cerebral vasomotor tone and blood–brain barrier integrity by dynamically buffering changes in CBF in response to spontaneous changes in MAP (Bailey *et al.* 2009*a*,*c*, 2011) justifying a complementary examination of the pressure–flow coupling dynamic. Contrary to our original expectations based on the published literature (Jansen *et al.* 2000), the elevated LF phase observed in CMS+ during spontaneous oscillations indicated improved pressure–flow coupling potentially related to the elevated CVR or hypocapnia as observed subsequent to changes in the respiratory chemoreflex (Ogoh *et al.* 2010) or, equally, to increased sympathetic tone (Lundby *et al.* 2018). The phase and gain findings associated with the elevated coherence during the driven oscillations revealed a similar mechanism, namely that gain was lower in the highlanders with the lowest values observed in CMS+. Lower gain is consistent with findings in Himalayan Sherpas (Smirl *et al.* 2014) and may reflect an improved ability to buffer perfusion during rapid alterations in BP to protect against vasogenic oedema in the face of exaggerated OXINOS. However, it would seem unlikely that this constitutes a functionally neuroprotective adaptation given that our participants, in particular CMS+, exhibited clinical signs of neurodegeneration (see below).

Clinical function

Researchers have suggested that the Andean model of HA living, defined by cerebral hypoperfusion and polycythaemia, is phenotypically maladaptive given that it carries a higher risk of stroke and migraine and is associated with increased morbidity and earlier mortality (Virues-Ortega *et al.* 2009, Jansen & Basnyat, 2011). In support of this, Bolivians born and bred at the same altitude and location to that used in the present study exhibited slower psychomotor speed in attention and digit symbol coding tasks that persisted across the lifespan, reflecting a 'speed–accuracy' trade-off such that slower may be surer (Hill *et al.* 2014). The present findings further extend the albeit limited literature identifying impairments in learning/memory and attention/information processing that were especially pronounced in CMS+.

Thus, it would appear that the highlander's brain, especially in CMS+, is unable to compensate for its hypoperfusion-induced CDO_2 constraint. Any global or

local cerebral O_2 deficit has the potential to impact cognition potentially due to impaired neurotransmitter release, which animal studies suggest is related to depressed monoamine synthesis (Freeman & Gibson, 1988), elevated glutamate excitotoxicity (Hota *et al.* 2008) and perturbations in choline acetyltranserase/acetyl cholinesterase expression (Guerra-Narbona *et al.* 2013).

The observation that clinical symptoms of depression were absent in CMS− and only apparent in CMS+, in whom cognitive/haemodynamic impairments were most pronounced, suggests that a physiological continuum for hypoxaemia-induced systemic OXINOS may potentially exist that when surpassed may prove maladaptive and contribute to the neurological complications associated with CMS. This hypothesis is not unreasonable given that hypoxia and OXINOS contribute to the pathophysiology of dementia (Sun *et al.* 2006; Wojsiat *et al.* 2018) and depression (Salim, 2014) in lowlander patient groups and extends our previous observations, albeit confined to the systemic vascular circulation (Bailey *et al.* 2013*c*). Alternatively, if systemic OXINOS/hypoxaemia are less pronounced it may simply take more time to develop clinical symptoms. Furthermore, our measurements obtained at rest may have underestimated the true magnitude of hypoxic stress encountered given that hypoxaemia in CMS+ is further compounded by physical activity (Stuber *et al.* 2010; Pratali *et al.* 2012) and sleep-disordered breathing (Rexhaj *et al.* 2016).

We observed a similar profile for performance on MoCA with consistently lower scores recorded in CMS+. Although originally developed as a measure of global cognitive function (Nasreddine *et al.* 2005), MoCA is frequently used as a clinical screening tool for the dementias (Ballard *et al.* 2013) with a cut-off score of 25 points or lower widely used as the threshold for detecting mild cognitive impairment and possible dementia (Davis *et al.* 2015*a*). Thus, with 87% of the CMS+ group fulfilling these clinical criteria, our findings may help identify those HA dwellers most 'at risk' and in need of more specialist neurological assessment to diagnose an emerging dementia syndrome. Clinical diagnosis is inherently complex, depending on the triad of cognitive function, patient report and informant history, notwithstanding complementary assessments of cerebrospinal fluid, blood-borne and structural biomarkers to exclude inflammatory, infective and malignancy-related causes of dementia (Burns & Iliffe, 2009; Robinson *et al.* 2015).

Experimental limitations

We need to consider several limitations when interpreting the present findings. First, the OXINOS assays we used, despite taking advantage of the most direct analytical techniques currently available, ultimately rely on *ex vivo* detection of relatively stable reactants confined to circulating plasma/red blood cells formed downstream of the primary source/reaction pathway that we assume reflects dynamic events *in vivo* (Bailey *et al.* 2009*a*). Given that these metabolites, especially GSH:GSSG, partition heterogenously across different tissues, our conclusions only apply to what we observed in circulating blood. In addition, there remains the inevitable translational challenge when attempting to determine the clinical outcomes associated with elevated OXINOS given our current inability to differentiate between physiologically adaptive and pathologically maladaptive concentration thresholds (Bailey *et al.* 2013*c*). Furthermore, we encourage interventional studies incorporating targeted antioxidant prophylaxis to disassociate cause from effect and confirm the mechanisms proposed herein. Second, we need to be cautious when interpreting the perfusion data in HA dwellers given that we relied on differences in MCAv as an indirect surrogate of global CBF that fails to take into account the antagonistic dilatory/constricting effects caused by prevailing hypoxia/hypocapnia (Wilson *et al.* 2011). Finally, future researchers need to consider more specialist follow-up neurological assessments to complement the current approaches taken to determine the prevalence of dementia in the most vulnerable CMS+ patient subgroup.

Conclusions

Notwithstanding the experimental limitations as outlined, these findings indicate that a chronic state of disequilibrium potentially exists between free radical formation and antioxidant defence in highlanders, causing systemic OXINOS to be permanently elevated and especially exaggerated in more hypoxaemic CMS+ patients. OXINOS was associated with blunted perfusion and reactivity to hypercapnia, impaired cognition and, in CMS+, symptoms of depression. Collectively, these findings are the first to suggest that a physiological continuum may exist for hypoxaemia-induced systemic OXINOS that when excessive is associated with accelerated cognitive decline and depression, helping identify those HA dwellers, especially CMS+, who may require more specialist neurological follow-up and targeted support. Future investigators need to consider the potential neuroprotective benefits of targeted antioxidant prophylaxis and cognitive training in this patient population. Finally, because arterial hypoxaemia is a hallmark feature of circulatory diseases, including those that affect the brain, the current findings in Aymaras may provide complementary insight into the pathophysiology and treatment of patients suffering from early-onset neurodegeneration at sea-level.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

DMB, US and CS conceived and designed the research. DMB, US and CS obtained funding. All authors contributed to data collection and analysis. All authors interpreted the results of the experiments. DMB drafted the manuscript and revisions thereof. All authors edited and revised the manuscript(s) and approved the final version submitted for publication. All authors agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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