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Biomarkers of hypoxia, endothelial and circulatory dysfunction among climbers in Nepal with AMS and HAPE: a prospective case–control study

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Abstract

Background: The mechanisms underlying acute mountain sickness (AMS) and high-altitude pulmonary edema (HAPE) are not fully understood. We hypothesized that regulators of endothelial function, circulatory homeostasis, hypoxia and cell stress contribute to the pathobiology of AMS and HAPE.

Methods: We conducted a prospective case–control study of climbers developing altitude illness who were evacuated to the CIWEC clinic in Kathmandu, compared to healthy acclimatized climbers. ELISA was used to measure plasma biomarkers of the above pathways.

Results: Of the 175 participants, there were 71 cases of HAPE, 54 cases of AMS and 50 acclimatized controls (ACs). Markers of endothelial function were associated with HAPE: circulating levels of endothelin-1 (ET-1) were significantly elevated and levels of sKDR (soluble kinase domain receptor) were significantly decreased in cases of HAPE compared to AC or AMS. ET-1 levels were associated with disease severity as indicated by oxygen saturation. Angiopoietin-like 4 (Angptl4) and resistin, a marker of cell stress, were associated with AMS and HAPE irrespective of severity. Corin and angiotensin converting enzyme, regulators of volume homeostasis, were significantly decreased in HAPE compared to AC.

Conclusion: Our findings indicate that regulators of endothelial function, vascular tone and cell stress are altered in altitude illness and may mechanistically contribute to the pathobiology of HAPE.

Key words: Acute mountain sickness, high-altitude pulmonary edema, hypoxia, endothelial dysfunction, circulatory homeostasis, endothelin-1, altitude illness

Introduction

Rapid ascent to altitudes of 2500 m and higher may result in altitude illness. Acute illness is often separated into three syndromes: acute mountain sickness (AMS), high-altitude cerebral edema (HACE) and high-altitude pulmonary edema (HAPE).[1–4] Individuals with AMS usually display symptoms of headache with/without fatigue, difficulty sleeping, dizziness and loss of appetite, nausea and vomiting. Patients with AMS may progress to HACE, defined by the onset of ataxia and/or altered consciousness, often as a result of continued ascent. HAPE is characterized by the onset of dyspnoea at rest, cough, decreased exercise tolerance and clinically by rales, wheezing, tachypnea and

tachycardia.[4–6] HAPE may occur by itself or in conjunction with AMS and HACE.

The precise pathophysiological mechanisms resulting in AMS, HAPE and HACE are not fully understood. Therefore, it is often difficult to identify those at risk of altitude illness and progression to HAPE or HACE in the absence of specific markers or models that predict individual susceptibility. A detailed understanding of the underlying pathobiology of these syndromes may identify predictive biomarkers and risk-stratification tools. The primary focus of this study was to therefore elucidate pathways underlying AMS and HAPE. Once validated, biomarkers of these pathways may have utility as predictive or diagnostic markers. It has been proposed that high-altitude hypoxemia induces both humoral and hemodynamic changes, including changes in sympathetic activity and endothelial function that ultimately culminates in pulmonary microvascular leak.[7–10] Hypoxia may also induce blood brain barrier dysfunction leading to endothelial cell junction disruption and cerebrovascular leak.[8,9]

HAPE is a non-cardiogenic hydrostatic pulmonary edema with marked increases in pulmonary artery and capillary pressure due to an exaggerated pulmonary vasoconstrictive response to hypoxia.[10,11] Nitric oxide (NO) plays a critical role in regulating endothelial function and vascular tone. Under hypoxic conditions, HAPE-susceptible individuals have reduced exhaled NO levels associated with increased pulmonary artery pressure and capillary leakage.[12–15] These data suggest that reduced NO biosynthesis may contribute to increased hypoxic pulmonary vasoconstriction.[16] Bioavailable NO has been reported to directly modify the angiopoietin (Ang)-Tie2 pathway, a major regulator of endothelial activation and microvascular leak. The angiogenic factors Ang-1 and Ang-2 play essential roles in endothelial function and integrity mediated through competitive binding of their cognate receptor Tie2.[17–20]

Regulators of circulatory homeostasis and blood volume, including the renin-angiotensin-aldosterone system, may play mechanistic roles in AMS and HAPE. Mutations in proteins controlling these pathways have been associated with performance and acclimatization at high altitudes.[21–23] Modulators of hypoxia and cell stress, such as resistin, may also have effects on susceptibility to AMS and HAPE. Resistin has been shown to induce apoptosis of pulmonary endothelial cells and to have pro-inflammatory effects that may contribute to pulmonary hypertension and HAPE.[8,24–26] Neuroprotective factors such as brain-derived neurotrophic factor (BDNF), have also been reported to be altered in individuals at high altitude and under hypoxic conditions.[27]

In this study, we used a case–control design to test the hypothesis that specific regulators and mediators of endothelial function, circulatory homeostasis and hypoxia may contribute to the pathobiology of AMS and HAPE.

Methods

Study Population

Between October 2010 and May 2011, we prospectively enrolled trekkers and climbers at the CIWEC Clinic Travel Medicine Centre in Kathmandu who were medically evacuated to the clinic with altitude illness. Data on patients were entered into the central database of the GeoSentinel Network according to a standardized protocol and were then extracted for this analysis.[28,29] This study was approved by the Kathmandu CIWEC regional IRB and participants provided informed consent. At enrolment, data were collected on the ascent profile, demographics, vital signs and a blood sample was collected and stored at $-20°C$ until biomarker analysis. A group of healthy acclimatized hikers/climbers acclimatized controls (ACs) was recruited as a control group. Among the 50 ACs, 29 were co-evacuated by helicopter with a case and co-presented to the clinic. The remaining 21 ACs were recruited from the waiting room, accompanying other climbers who presented with illnesses other than AMS or HAPE. Altitude illness was assessed according to Lake Louise definitions of altitude illness.[30] Subjects were classified as having altitude illness if they met the Lake Louise definitions of AMS or HAPE.[31]

Biomarker Assessment

Biomarkers from selected pathways implicated in the pathogenesis of altitude illness were measured in EDTA plasma by ELISA (Quantikine or DuoSets, R&D Systems, Minneapolis, MN) using dilution factors: Endothelin-1 (ET-1;1:2), sE-Selectin (1:5), Ang-2 (1:2), Ang-1 (1:10), soluble intercellular adhesion molecule-1 (sICAM-1;1:100), soluble receptor tyrosine kinase Tie2 (sTie-2;1:25), soluble endoglin (sEng;1:25), soluble kinase insert domain receptor (sKDR;1:10), angiopoietin-like 4 (Angptl4;1:5), renin (1:5), angiotensin-converting enzyme (ACE;1:25), corin (1:5), cystatin C (1:1000), BDNF (1:25), resistin (1:25) and interleukin-1 receptor antagonist (IL-1Ra;1:2) as described.[20,32–34]

Statistical Analysis

Statistical analyses were performed using GraphPad Prism v.6.04 and IBM SPSS v.20. All analyses were non-parametric. Comparisons between continuous variables were performed using Mann–Whitney U test with Bonferonni adjustment. Binary outcomes were analysed using Chi-Square or Fisher's exact test, and correlations were investigated using Spearman's rho.

Results

Description of Study Population

A total of 175 consecutive consenting participants were enrolled in this case–control study to evaluate host response biomarker profiles in cases with altitude illness ($n = 125$) compared to ACs ($n = 50$). Baseline characteristics were comparable between the ACs and individuals with altitude illness with median [interquartile range (IQR)] ages of 40.0 (30.0–53.0) and 43.0 (32.0–56.5) years, respectively ($P = 0.21$). In the ACs, 50% were male compared to 62.4% in cases of altitude illness ($P = 0.13$). There was no difference in the maximum altitude attained between the two groups with ACs reaching a median altitude of 4240 m (IQR, 3870–5300) and those who developed altitude illness reaching a maximum altitude of 4410 m (3770–4930) ($P = 0.57$). Acetazolamide use by the study population is shown in Figure 1. Four of the ACs reported taking acetazolamide for prophylaxis, whereas 42.4% ($n = 53$) of cases reported using acetazolamide but primarily for treatment with 43.4% of cases starting acetazolamide within 24 h before presentation.
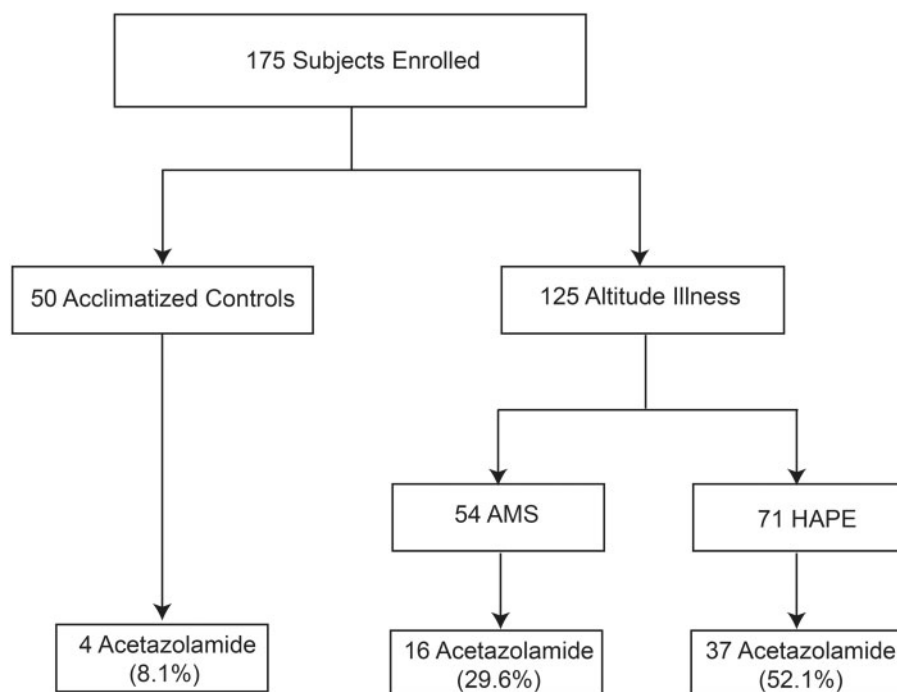
Figure 1. Flowchart of study subjects evaluated for biomarkers of altitude illness

Table 1. Characteristics of climbers with altitude illness

Characteristic	AMS ($n = 54$)	HAPE ($n = 71$)	P
Age (years)	37.0 (27.0–55.8)	47.0 (37.0–57.5)	0.054
Sex (M), n (%)	28 (51.9)	50 (70.4)	0.034
Maximum altitude (m)	4310 (3550–4930)	4410 (3860–4983)	0.397
Percentage took Diamox[a]	16 (29.6)	37 (52.1)	0.012
Temperature (°C)	37.0 (36.6–37.3)	37.4 (37.0–38.0)	0.001
Systolic BP (mm Hg)	120 (102–130)	110 (100–130)	0.248
Pulse rate (bpm)	75.0 (64.5–82.0)	80.0 (74.0–89.5)	0.034
SpO_2 (%)	97.0 (95.0–98.0)	91.0 (85.5–96.0)	< 0.001
WBC	9000 (7100–11 600)	11 150 (7975–14 188)	0.064
Hematocrit (%)	43.5 (40.8–48.0)	43.0 (42.0–46.0)	0.943
Lake Louise Score	11 (9–13)	14 (11–16)	0.002

Measurements of characteristics were taken upon evacuation/presentation to the clinic for each climber and analysed by Mann–Whitney U test.

[a]Diamox taken for treatment of symptoms.

Of the 125 cases of altitude illness, 54 had AMS, 57 had HAPE and 14 were classified as both AMS and HAPE (considered HAPE for this analysis). HAPE was the predominant manifestation observed and therefore the focus of this study. Of the subjects with HAPE, 29 also had HACE but were classified as HAPE for this analysis. There were no significant differences observed in the maximum altitude reached between the AMS and HAPE groups (median, IQR: 4310 m, 3550–4930 in AMS vs. 4410 m, 3860–4983 in HAPE, $P = 0.40$). Overall, individuals who developed HAPE were more likely to be male and were older than those with AMS (Table 1).

Biomarkers Associated with Altitude Illness

We initially compared biomarkers from three pathways implicated in the pathobiology of altitude illness in all participants who developed altitude illness ($n = 125$) versus ACs (Table 2). Three markers of endothelial activation and vascular permeability were significantly different between cases of altitude illness and ACs; ET-1 ($P = 0.006$) and angptl4 ($P = 0.002$) were significant elevated, while sKDR was significantly decreased ($P < 0.001$). These markers remained significant after correcting for multiple comparisons. Three markers of circulatory homeostasis were significantly different when comparing individuals with altitude illness with ACs; cystatin C was significantly elevated ($P = 0.018$), while corin and ACE were significantly decreased ($P = 0.018$; $P = 0.036$, respectively). Resistin, a marker of hypoxia and cell stress was significantly elevated when comparing cases of altitude illness with AC ($P < 0.0001$; Table 2).

We next explored the association between the biomarkers and pulmonary manifestations of altitude illness by comparing AMS versus HAPE cases (Figure 2). There were three main

Table 2. Biomarker levels in acclimatized controls and climbers who developed altitude illness

Biomarker	Healthy acclimatized controls, $n = 50$	Altitude illness, $n = 125$	P
ET-1	1.42 (1.09–1.81)	1.76 (1.27–2.41)	0.006
sE-Selectin	18.42 (14.67–25.90)	19.86 (13.82–24.93)	0.492
Ang-2	0.53 (0.37–1.12)	0.79 (0.48–1.25)	0.063
Ang-1	11.95 (4.01–27.33)	12.20 (5.71–24.57)	0.887
sICAM-1	108.75 (95.68–132.30)	111.80 (97.15–127.30)	0.865
sTie2	16.48 (11.68–20.46)	15.93 (12.39–18.55)	0.490
sEng	10.0 (8.7–12.2)	9.15 (7.34–11.59)	0.124
sKDR	5.44 (4.42–6.69)	4.69 (3.89–5.73)	0.001
BDNF	9.9 (2.9–18.7)	8.38 (3.38–16.28)	0.703
Angptl4	50.4 (40.6–68.4)	65.94 (51.63–88.00)	0.002
Resistin	8.7 (6.4–13.8)	12.43 (8.99–16.04)	<0.0001
sIL-1Ra	0.28 (0.07–0.67)	0.29 (0.10–0.86)	0.417
Corin	1.01 (0.63–1.25)	0.73 (0.45–1.10)	0.018
ACE	91.6 (80.0–123.8)	88.18 (64.89–109.35)	0.036
Cystatin C	832 (733–983)	910.0 (792.0–1062.0)	0.018
Renin	0.28 (0.17–0.38)	0.29 (0.19–0.43)	0.666

BDNF, brain-derived neurotrophic factor. Data expressed as median (IQR), in ng/ml, analysed by Mann–Whitney *U* test.

observations: (i) biomarkers that were significantly elevated in both AMS and HAPE. Compared to ACs, Angptl4 and resistin were elevated in AMS ($P = 0.005$; $P = 0.001$, respectively) and HAPE ($P = 0.007$; $P = 0.001$, respectively) and remained significant after correcting for multiple comparisons. (ii) Biomarkers associated with HAPE alone. ET-1 was elevated ($P < 0.001$) and sKDR was decreased ($P < 0.001$) in ACs and AMS versus HAPE and remained significant after correcting for multiple comparisons. (iii) Biomarkers decreased in HAPE compared to ACs. Corin and ACE were decreased in HAPE compared to ACs ($P = 0.002$; $P = 0.003$, respectively) and remained so after correcting for multiple comparisons (Figure 2).

Furthermore, we assessed the impact of acetazolamide on biomarker levels from cases. Corin and sKDR were decreased ($P = 0.003$, $P = 0.023$, respectively) in those who reported acetazolamide use (42.4%).

Relationship between Biomarker Levels and Physiological Measures of Severity

We further explored the relationship between biomarkers and physiological and laboratory measures of disease severity to gain insight into possible mechanisms of disease pathogenesis. ET-1 and sKDR were associated with total leukocyte count (WBC) and peripheral oxygen saturation (SpO_2). Increased WBC count was positively correlated with ET-1 (rho = 0.287, $P = 0.03$) and negatively with sKDR (rho = −0.236, $P = 0.028$), while SpO_2 was negatively correlated with ET-1 (rho = 0.336, $P = 0.02$) and positively with sKDR (rho = 0.227, $P = 0.043$). sE-Selectin, a marker of endothelial activation, was positively correlated with pulse rate (rho = 0.333, $P = 0.04$) and negatively correlated with the SpO_2 (rho = −0.365, $P = 0.03$). There was a negative correlation between angptl4 and SpO_2 (rho = −0.335, $P = 0.002$).

Discussion

In this study, we investigated a panel of biomarkers reflective of pathways implicated in the pathobiology of altitude illness, focusing on pathways associated with endothelial dysfunction and

for which novel therapeutics are under development (e.g. Ang/Tie2 pathway).[32–34] We show that markers and mediators of endothelial activation, circulatory homeostasis and hypoxia are altered in individuals who develop AMS and HAPE compared to healthy acclimatized climbers. We report that increased circulating ET-1 levels were associated with HAPE and measures of disease severity including leukocyte count and SpO_2. In contrast, increased systemic sKDR levels were negatively correlated with HAPE and measures of disease severity. Collectively, these observations suggest that these regulators of pulmonary vascular function may mechanistically contribute to the pathobiology of HAPE, correlate with disease severity and may be clinically informative.

Our findings are supported by previous studies showing a role for ET-1 in HAPE and a direct correlation of ET-1 levels with pulmonary artery pressure at high altitudes.[35–37] ET-1 is synthesized by the pulmonary endothelium and has been reported to circulate at increased concentrations at high altitudes.[37] ET-1 is a key regulator of vascular tone and renal homeostasis and increased plasma levels are associated with pulmonary hypertension and support our findings of an association with HAPE and disease severity.[38,39] ET-1 is thought to be regulated via the transcription of ET-1 gene (*edn1*) and exocytosis of endothelial Weibel–Palade bodies (WPB).[40,41] NO controls WPB exocytosis and the expression of *edn1* but NO is reported to be reduced in individuals that are susceptible to HAPE.[14,40–42] These observations fit a model whereby reduced bioavailable NO would be expected to result in increased WPB exocytosis, release of Ang-2, endothelial dysfunction and higher levels of circulating ET-1. Collectively, these events may exacerbate hypoxic pulmonary vasoconstriction and increase the risk of HAPE.[37,43]

sKDR (also known as VEGF receptor 2) is the soluble truncated variant of KDR expressed by endothelial cells that binds to and inhibits vascular endothelial growth factor (VEGF), a potent inducer of microvascular leak.[44,45] The lower levels of circulating sKDR we observed in cases of HAPE is consistent with the hypothesis that there is less sequestration of VEGF and therefore more free local VEGF to mediate pulmonary vascular leak.
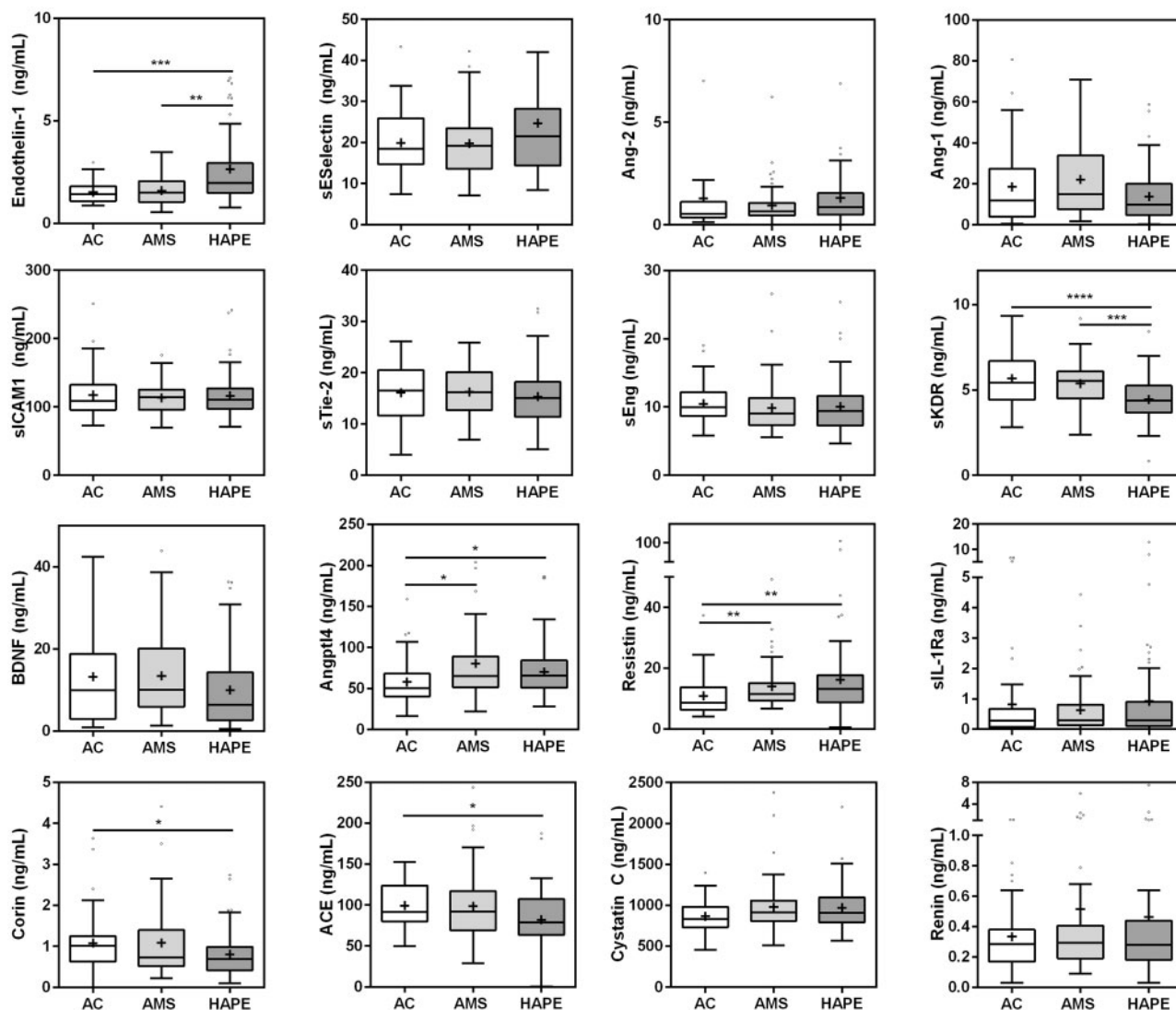
Figure 2. Box and whisker plots showing the distribution of biomarker levels in climbers returning from altitude. The box represents the median, and IQR, while the whiskers denote the 25 percentile minus 1.5IQR and 75 percentile plus 1.5IQR. Individual data points that fall beyond the whiskers are represented by dots. The group mean is shown by the (+). Data were analysed using the Kruskal–Wallis test with Dunn's multiple comparisons test comparing acclimatized healthy controls (AC) vs. AMS, AMS vs. high altitude pulmonary edema (HAPE) and AC vs. HAPE. The stars represent significant comparisons by post-hoc testing (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$)

Angptl4 is a hormone involved in glucose and lipid metabolism that is induced under hypoxic conditions.[46,47] Angptl4 has been proposed to promote vascular leak through integrin-mediated signalling or via hypoxia-induced apoptosis.[48,49] In this study, an increase in Angptl4 levels was associated with altitude illness and negatively correlated with SpO$_2$. Further study will be required to determine if Angptl4 plays a mechanistic role or is merely reflective of hypoxia.

There is considerable evidence supporting a causal role for the Ang-Tie2 pathway in regulating microvascular leak in acute lung injury and other conditions that share pathophysiologic features with HAPE.[50–55]. Ang-1 promotes endothelial quiescence and stability, whereas Ang-2 completes for Tie2 binding and promotes endothelial activation and permeability.[14,56] In this study, there were alterations in the Ang-Tie2 axis suggesting a relationship between HAPE and increased circulating Ang-2 and decreased Ang-1 levels associated with HAPE. However, the associations were not strong, perhaps reflecting that the

kinetics of markers of this pathway were not well suited to the timing of sample acquisition in this study. This hypothesis will need to be further investigated in larger prospective studies with sample collection closer to the onset of HAPE.

In this study, markers of circulatory homeostasis were associated with altitude illness and severity. Decreased levels of ACE were associated with HAPE compared to AC. ACE is expressed mainly on the lung endothelium and kidney epithelium and converts angiotensin I into physiologically active peptide angiotensin II, which acts as a potent vasopressor, controlling blood pressure and fluid electrolyte balance. Of note, polymorphisms in the *ACE* gene have been associated with successful acclimatization to extreme altitudes, increased transcription of ACE and with AMS/HAPE susceptibility in some ethnic backgrounds[57–60] but not others.[21,22,61] This study is consistent with the hypothesis that an increase in circulating ACE levels are associated with protection from developing HAPE.

Corin is a serine protease that converts pro-ANP into active ANP, regulating blood pressure and volume.[62] Active ANP is required to reduce sodium levels, resulting in lower blood pressure. In this study, corin levels were significantly lower in cases of HAPE, consistent with studies that have linked SNPs in human corin that reduce activity or expression, to an increased risk of hypertension or preeclampsia.[63–67]

One marker of hypoxia and cell stress, resistin, promotes pulmonary endothelial cell apoptosis and has been shown to be up-regulated in hypoxia-induced pulmonary hypertension models.[26,68,69] In this study, higher plasma resistin levels were associated with both AMS and HAPE, but have not with disease severity.

This study has limitations including a 1–2 days delay in sample acquisition from disease onset in some cases and limited data on the rate of ascent. However, the time from maximum altitude to blood draws was comparable between groups ($P > 0.05$; Table 1). Ideally samples should be collected as soon after the onset of symptoms of AMS and HAPE (i.e. before evacuation); however, this presents some challenging logistical constraints. Studies suggest that the rate of ascent is correlated to acclimatisation. Therefore, to test the hypothesis that genetic and physiologic susceptibility are risk factors for developing altitude illness, future studies should ideally examine cases and controls that are on identical ascent schedules. Future studies will also need to confirm and extend these findings by following climbers through their ascent, monitoring the kinetics of biomarkers of endothelial function, vascular homeostasis, hypoxia and cell stress during the onset and progression of altitude illness, as well as investigating polymorphisms in the genes that underlie these pathways.

In summary, this study focused on the association of altitude illness with pathways of microvascular leak, circulatory homeostasis and hypoxia. An improved understanding of mechanistic pathways that underlie life-threatening manifestations of altitude illness such as HAPE may help ultimately identify predictive markers of disease severity and outcome and putative new interventions (i.e. pro-ACE, pro-corin, pro-Ang-1 or anti-ET-1 approaches) to improve clinical outcome.

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