Exhaled volatile organic compounds in individuals with a history of high altitude pulmonary edema and varying hypoxia-induced responses

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Abstract

With ascent to altitude, certain individuals are susceptible to high altitude pulmonary edema (HAPE), which in turn can cause disability and even death. The ability to identify individuals at risk of HAPE prior to ascent is poor. The present study examined the profile of volatile organic compounds (VOC) in exhaled breath condensate (EBC) and pulmonary artery systolic pressures (PASP) before and after exposure to normobaric hypoxia (12% O₂) in healthy males with and without a history of HAPE (Hx HAPE, n = 5; Control, n = 11). In addition, hypoxic ventilatory response (HVR), and PASP response to normoxic exercise were also measured. Auto-regression/ partial least square regression of whole gas chromatography/mass spectrometry (GC/MS) data and binary logistic regression (BLR) of individual GC peaks and physiologic parameters resulted in models that separate individual subjects into their groups with variable success. The result of BLR analysis highlights HVR, PASP response to hypoxia and the amount of benzyl alcohol and dimethylbenzaldehyde dimethyl in expired breath as markers of HAPE history. These findings indicate the utility of EBC VOC analysis to discriminate between individuals with and without a history of HAPE and identified potential novel biomarkers that correlated with physiological responses to hypoxia.

1. Introduction

High altitude pulmonary edema (HAPE) is a non-cardiogenic pulmonary edema that can occur in non-acclimatized, healthy and often young individuals within 2–4 d of rapid ascent to altitudes greater than 2500 m above sea level (Hackett 1999) and is the most common cause of death at high altitude. The origin and pathogenesis of HAPE and the factors that render certain individuals more susceptible to developing HAPE are poorly understood. Previous research has shown that there may be multiple factors that indicate a history of HAPE, including an exaggerated hypoxic pulmonary vasoconstriction (HPV), blunted hypoxic ventilatory response (HVR) and abnormal increases in pulmonary arterial pressures during exercise at sea level (Matsuzawa et al 1989, Hohenhaus et al 1995, Eldridge et al 1996, Grunig et al 2000, Dehnert et al 2005, Swenson and Bartsch 2012). Individuals who have a history of HAPE have HPV responses that are much greater than individuals without a history of HAPE, as shown by greater increases in pulmonary arterial pressures and pulmonary vascular resistances when exposed to significant hypoxia and/or hypobaria (Hultgren et al 1971, Kawashima et al 1989, Grunig et al 2000). Additionally, individuals with a history of HAPE have a significantly reduced isocapnic HVR when compared to individuals without a history
of HAPE (Matsuzawa et al. 1989, Hohenhaus et al. 1995) that could lead to a reduced alveolar PO₂ and possibly contribute to a greater HPV (Swenson and Bartsch 2012). Investigators (Kawashima et al. 1989, Eldridge et al. 1996, Grunig et al. 2000, Dehnert et al. 2005) have also found that individuals with a history of HAPE have greater exercise-induced increases in pulmonary arterial pressures at sea level than their non-history of HAPE counterparts as shown both by catheterization (Eldridge et al. 1996) and stress Doppler echocardiography studies (Grunig et al. 2000).

The methods for measuring HVR, HPV and pulmonary vascular response to exercise are difficult to perform and equipment-intensive, especially in the field, and are not always reliable predictors of HAPE history or susceptibility (Selland et al. 1993). We recently used expired breath condensate (EBC) analysis of volatile compounds in an effort to discern severe pulmonary arterial hypertension patients from healthy controls (Mansoor et al. 2014). Using this method, volatile chemical compounds are identified in the breath using gas chromatography mass spectrometry (GC/MS); the metabolites found in EBC are often different between the groups and/or the amounts vary between the groups (de Lacy Costello et al. 2014). The purpose of this study was to use EBC analysis to distinguish between individuals with a history of HAPE and controls before and after 90 min exposure to 12% oxygen. The ability to distinguish HAPE susceptibility using EBC analysis could lead to a simple, non-invasive field test.

2. Materials and methods

2.1. Ethics statement

This study was approved by the University of California, Davis, Office of Human Research Protection Institutional Review Board (protocol #200917422-2). Written informed consent was obtained from participants and the study was conducted according to principles expressed in the Declaration of Helsinki.

2.2. Subjects

Sixteen healthy, actively exercising, nonsmoking, adult male volunteers, ages 18–35, participated in the study (table 1). Subjects were divided into two groups based on prior altitude experience. Subjects who had experienced an episode of high altitude pulmonary edema (HAPE) were placed in the history of HAPE group (Hx HAPE, n = 5) (table 2). The control group (N = 11) was composed of individuals who had minimal to no experience traveling to altitudes in excess of 2440 m and had never experienced HAPE. Pulmonary function tests were performed via a digital spirometer (Futuremed, Spirovision) and the subjects were medically cleared by a pulmonologist.

2.3. Research design and data collection

On the first day of the study, subjects performed a hypoxic ventilatory response (HVR) test after which they had a 20 min rest, followed by a peak oxygen uptake test on a recumbent bicycle at the University of California, Davis in Davis, California. On the following day at the University of California, Davis Medical Center in Sacramento, California, expired breath samples were taken from the subjects, followed by an exercise stress test with echocardiography under normoxic conditions. About 15 min after the exercise, the subjects rested in the supine position for ninety minutes while breathing a hypoxic gas mixture of 12% O₂, followed by post-hypoxia expired breath and sample collection.

2.3.1. Hypoxic ventilatory response (day 1)

The HVR protocol was a modified version of Levine et al. (1992) and Hohenhaus et al. (1995) measuring isocapnic hypoxic ventilatory responses. Subjects rested in a dark room for 30 min prior to the test wearing a facemask with a sample line connected to a portable metabolic cart (Oxycon Mobile, Jaeger, CareFusion Corporation) for measurement of minute ventilation (VE), partial pressure of end-tidal CO₂ (PETCO₂) and VO₂. Subjects inspired a mixture of compressed air and variable amounts of nitrogen and CO₂ from a 12L mixing chamber where the oxygen concentration was decreased by approximately 0.5% every 30 s as measured by an oxygen analyzer (Ceramatec, model M-25MEL). PETCO₂ remained at approximately 36.68 mm Hg ± 2.60 mm Hg during the test. The procedure was stopped when the subject’s arterial oxygen saturation reached 80% (finger pulse oximeter: Nellcor Puritan, model NPB-40). The HVR was then quantified by plotting minute ventilation (V̇E) against 1 minus the oxygen saturation (SaO₂).

2.3.2. Peak oxygen uptake (day 1)

Subjects performed a peak oxygen uptake test on a recumbent bike (Cateye ergociser EC-3500) approximately 20–30 min after the HVR procedure.

| Table 1. Subject characteristics. |
|---------------------------------|
|                                | Control (n = 11) | History of HAPE (n = 5) |
| Age (years)                    | 25.0 ± 4.02     | 29.2 ± 6.94              |
| Height (cm)                    | 181.42 ± 4.33   | 183.79 ± 4.36            |
| Weight (kg)                    | 81.24 ± 10.10   | 84.55 ± 7.43             |
| FVC (L)                        | 6.06 ± 0.52b    | 6.10 ± 0.83b             |
| FEV₁ (L)                       | 4.81 ± 0.51b    | 4.96 ± 0.81b             |
| FEV₁/FVC (%)                   | 79.59 ± 8.23b   | 81.35 ± 6.30b            |
| Absolute peak VO₂              | 3.85 ± 0.39     | 3.90 ± 0.49              |
| (L/min⁻¹)                      |                  |                          |
| Relative peak VO₂              | 47.62 ± 6.55    | 47.82 ± 8.01             |
| (L/min⁻¹×kg⁻¹)                 |                  |                          |
| HVR (V̇E×SaO₂)                 | 1.74 ± 0.88     | 0.82 ± 0.57b             |

*a p = 0.054.

b All subjects within normal age predicted range.

FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; HVR, hypoxic ventilatory response.

Note: Values are means ± SD.
The subjects wore a facemask attached to a sampling line and rotary flow sensors and a vest which held the batteries and metabolic data acquisition instruments that interfaced with a PC computer via telemetry (Oxycon Mobile, Jaeger, CareFusion Corporation). Oxygen uptake (VO₂), end-tidal O₂ and CO₂, VE, respiratory exchange ratio, breathing frequency and tidal volume were obtained. Subjects also wore a heart rate monitor. The subjects pedaled at 75 rpm and workload started at 50 W and was increased by 50 W every two minutes. The test was stopped after 16 min (400 W) or when the subject could no longer maintain 75 rpm (voluntary fatigue). Heart rate and rating of perceived exertion (0 = light work; 10 = heavy work) was recorded at the end of every two-minute stage.

2.3.3. Exercise stress test with Doppler echocardiograph (day 2)
Subjects laid supine on a gurney that tilted laterally 20–30 degrees and was connected to a variable load supine exercise cycle ergometer (Stress Echo Bed, Medical Positioning, Inc.). The subjects were prepped with a three lead ECG and a facemask interfaced with a metabolic data acquisition system (Oxycon Mobile, Jaeger, CareFusion Corporation). 2D Doppler echocardiographic recordings (Acuson Sequoia C512 Echo Machine) to estimate pulmonary artery systolic pressures (PASP) similar to Yock and Popp (1984) were obtained every two minutes as the subjects exercised at 75 rpm starting at a workload of 25 W and increased by 25 W every two minutes. Exercise ended due to exhaustion (n = 2, 175 W), insufficient tricuspid regurgitant signals (n = 2, 150 W) or at a workload of 200 W (n = 12). Estimates of PASP were obtained using experienced cardiac sonographers measuring tricuspid valve regurgitant jet velocity in triplicate at rest and every two minutes during exercise.

Table 2. Subject history of HAPE.

| Subject | Mountain location | Highest elevation reached (m) | History | Symptoms |
|---------|------------------|-----------------------------|---------|----------|
| 1       | Pico de Orizaba (5636) Mexico | 5030 | 3 d to 5030 m, felt symptoms; descended to 4267 m | Cough; dyspnea; crackles; frothy sputum |
| 2       | Cotopaxi (5897) Ecuador | 5790 | 3 d to 5790 m, felt symptoms; descended to 3048 m | Cough; fatigue; dyspnea; bloody sputum |
| 3       | Pico de Orizaba (5636) Mexico | 5180 | 3 d to 5180 m, felt symptoms; descended to 3048 m | Cough; nausea; headache; dyspnea; pink frothy sputum |
| 4       | Aconcagua (6960) Argentina | 4500 | 3 d to 4500 m, felt symptoms; descended to 2500 m | Dehydration; diarrhea; fatigue; gurgling rales |
| 5       | Aconcagua (6960) Argentina | 5180 | 6 d to 5180 m, felt symptoms; descended to 4267 m | Fatigue; dizziness; dyspnea; gurgling rales |

The facemask was connected to a three-way valve that was attached via plastic hosing to a 30 L Douglass Bag filled with a gas mixture of 12% oxygen and 88% nitrogen (Grunig et al 2000) and monitored using an oxygen analyzer (Ceramatec: Model OM-25ME). Subjects breathed the hypoxic gas mixture for 90 min while awake but calm, with oxygen saturation (Nellcor Puritan: Model NPB-40) and heart rate monitored continuously and recorded every five minutes. Pulmonary artery systolic pressure was measured as stated above at the start and every 15 min during the hypoxic exposure, as were Lake Louise scores (Savourey et al 1995).

2.3.5. Expired breath condensate (EBC) collection (day 2)
Expired breath condensate samples were collected before and after the 90 min hypoxic exposure and analyzed in the same manner as in Mansoor et al (2014). Briefly, subjects sat quietly in a chair with a nose clip on and breathed through a mouthpiece connected to the Jaeger EcoScreen (Viasys Healthcare, Conshohocken, PA) breath condensate system for about 20 min. Approximately 0.5 mL of EBC sample was then stored at −80°C and later analyzed for volatile organic compounds using solid-phase micro-extraction (SPME; Supelco, St. Louis, MO) followed by gas chromatography mass spectrometry analysis (GC/MS; Varian 3800 GC with an electron ionization 4000 Ion Trap MS, Varian, Walnut Creek, CA).

2.4. Data analysis
All statistical analyses were performed using SPSS software version 22 (IBM Corp, Armonk, NY) unless specifically stated.

2.4.1. Anthropometric and physiologic variables
Anthropometric variables plus the HVR were compared using a MANOVA to determine if there were statistical differences between the groups. Equality of variances was determined using Levene’s test. Group PASP response to exercise was analyzed using a MANOVA
with input variables of percent of peak VO₂ and PASP at each workload up to 100 watts. Group PASP, SaO₂, heart rate and Lake Louise Scores during hypoxia were analyzed using a mixed linear model with time treated as a repeated measure. In addition, the group mean responses of PASP and SaO₂ over the last 30 min of the hypoxia exposure were compared using MANOVA.

2.4.2. Gas chromatograms/mass spectra peak analysis
The samples were analyzed as described previously by Mansoor et al (2014). Briefly, the 1 ml aliquot of collected EBC sample was placed in a borosilicate vial; 1 ml of saturated NaCl solution was added to reduce solubility of dissolved VOCs. The vials were then heated to 90°C and VOCs sampled using Carboxen/Polydimethylsiloxane SPME fibers. The adsorbed chemical were desorbed in the injector port of the Varian 3800 GC at 250°C and analyzed by GC/MS. Instrument performance was controlled by injecting a standard Grob DA 280 Column Test Mix (Restek, Bellefonte, PA). The collected data were then routed for numerical analysis. The EBC collection apparatus does not allow for straightforward introduction of a standard during the sample collection stage. In order to ensure minimal sample-to-sample variation and avoid potential bias, GC/MS analysis for all samples was carried out as a single batch.

2.4.3. Discriminatory analysis
Pre- and post-hypoxia GC/MS data were converted into total ion count versus time. Each GC profile was composed of approximately 15 000 time points that covered 255 min. Baseline correction was applied to remove humps/plateaus in some of the chromatograms (Andrade and Manolakos 2003). Auto-regression (AR) analysis was then used to reduce the number of variables needed to describe chromatograms from the original time scan number to one hundred AR coefficients (Zhao et al 2008, Zhao and Davis 2009). To visually and quantitatively compare the chromatography data of control and Hx HAPE subjects, partial least square regression (PLSR) using AR coefficients was employed. Partial least square regression further reduced the dimensionality from one hundred AR coefficients to two latent variables (PLSR components). The two PLSR components were then plotted against each other to examine separation within the data. This analysis was performed using Matlab software (MathWorks, Inc.; Natick, MA). The pre- and post-hypoxia models were validated using the leave-one-out validation process. The results of this validation were used to calculate the overall separation accuracy (Mansoor et al 2014).

In order to further explore separation within the data we examined the measured physiological responses to hypoxia, the heights of individual peaks with the pre- and post-hypoxia GC/MS chromatogram and the hypoxia-induced change (post minus pre) in peak heights using forward stepwise binary logistic regression (BLR). Stepwise selection for variable inclusion was based on the significance of the score statistic, while variable removal was based on the probability of a likelihood-ratio statistic that is based on the maximal partial likelihood estimates. Goodness-of-fit for the models generated was determined using the Hosmer-Lemeshow test. The results of the BLR were used to calculate the overall separation accuracy.

2.4.4. Correlation analysis
Mean responses of PASP and SaO₂ over the last 30 min of the hypoxia exposure were correlated with all normalized pre-hypoxia peak heights and hypoxia-induced changes in peak heights using Pearson-product moment correlation. Correlation analysis was followed by stepwise linear regression using pre-hypoxia peak heights of individual compounds against mean responses of PASP and SaO₂ over the last 30 min of the hypoxia exposure in order to identify potential predictors of the hypoxic physiologic response. Additionally, stepwise linear regression analysis was done using the change in peak heights against mean responses of PASP and SaO₂ over the last 30 min of the hypoxia exposure.

2.4.5. Chemical Identification
Chemical identities of the 32 peaks were explored. Where necessary, the Automated Mass Spectral Deconvolution and Identification System (AMDIS) GC/MS analysis software (National Institute of Standards and Technology [NIST] v.2.64) was used to remove background noise and deconvolve peaks for co-eluting compounds. The MS spectra fragmentation patterns and Kovats indices were compared against the NIST 2005 and Wiley 2009 MS libraries of deconvolved peaks for coeluting compounds. The MS spectra and Kovats indices were compared against the NIST 2005 and Wiley 2009 MS libraries of deconvolved peaks for co-eluting compounds using NIST Mass Spectral Search Software v.2.0. The highest probability matches were considered and putative chemical identity was determined empirically by examining representative MS data and m/z in the data set. If the search produced...
a match with a probability greater than 80%, that match was considered to be the unknown compound (high confidence match). In some cases, no match was found or multiple chemical matches with very similar mass spectral fragmentation patterns and close match probability values (e.g. for isomers) were found. These were considered to be low confidence matches (Mansoor et al. 2014).

3. Results

3.1. Subject characteristics and hypoxic ventilatory response
All subjects were healthy at the time of the study. Group mean values (± standard deviation) for age, height, weight, forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), FEV1/FVC and absolute and relative peak VO2 are given in table 1. There were no statistically significant differences in any of these variables between the two groups. Pulmonary function measurements for all subjects were within normal age-predicted range (FVC = 104.4 ± 8.7%; FEV1 = 101.8 ± 9.5%; FEV1/FVC = 97.8 ± 8.9%). There was, however, a significant difference between groups for HVR, with the Hx HAPE group having a blunted response (table 1 and figure 1). Table 2 shows the history and symptoms of HAPE experienced by the Hx HAPE group.

3.2. Exercise with Doppler echocardiography
The PASP response to increasing workload is shown in figure 2. Both PASP and percent of peak VO2 increased significantly with increasing workload from 0 to 100 W (PASP, p = 0.028; percent of peak VO2, p = 0.000), however, there was no significant difference between the control and Hx HAPE groups for the PASP response to increasing workload (p = 0.997).

3.3. Ninety minute hypoxic exposure with Doppler echocardiography
With exposure to hypoxia, both the control and Hx HAPE group had significant (p < 0.001) increases in PASP (figure 3(a)). There was a significantly greater PASP (p < 0.05) at 45, 60 and 75 min of hypoxia in the Hx HAPE group compared to the control group (figure 3(a)). In addition, the mean PASP over the last 30 min of the hypoxic stress was significantly greater in the Hx HAPE group compared to the control group (p = 0.019).

With exposure to hypoxia there was a significant (p < 0.001) decrease in SaO2 overall (figure 3(b)). There were no significant differences in SaO2 between the control and Hx HAPE groups at any time point during the hypoxic exposure. Also, there were no significant differences in heart rate overall or between groups during the hypoxic exposure. (figure 3(c)). With exposure to hypoxia there was a significant (p < 0.001) increase in modified Lake Louise Scores overall (figure 3(d)), but there were no significant differences in modified Lake Louise Scores between the control and Hx HAPE groups.

3.4. Whole GC/MS chromatogram and individual peak analysis
Figure 4(a) shows an overlay comparison of the gas chromatogram of a pre-hypoxia EBC sample from a control subject (gray line) and a HAPE subject (black line). Figure 4(b) compares the pre-hypoxia gas chromatogram (gray line) of the same control subject with their post-hypoxia gas chromatogram (black line). Three peaks with retention times of 65.266, 97.754 and 117.488 min were significantly different between the control and Hx HAPE groups. Figures 5(a) and (b) shows the plotted results of AR/PLSR analysis of GC peaks for the pre- and post-hypoxia exhaled breath condensate samples. The results of the AR/PLSR and
Figure 3. Response of pulmonary artery systolic pressure (PASP), arterial hemoglobin saturation (SaO₂), heart rate (HR) and modified Lake Louise Score to 90 min supine resting normobaric hypoxic exposure in HAPE and control groups.
BLR analyses are compared in table 3. While the plot of the PLSR coefficients suggest good separation, the calculated overall separation accuracy for the pre- and post-hypoxia EBC samples was 50 and 68.8 percent, respectively. In contrast, BLR analysis of both the physiologic response and pre-hypoxia peak height data resulted in models with overall separation accuracy of 100 percent, as was true when all of the data were combined. BLR analysis using post-hypoxia or the change in peak height with hypoxia resulted in models with lower overall separation accuracy (87.5 and 93.8), but was still better than the AR/PLRS results. Interestingly, the abundance of the compound with peak at a retention time of 97.75 min in the pre-hypoxia EBC sample and the abundance of the compound with peak at a retention time of 116.40 min in the pre- and/or post-hypoxia EBC sample were consistently selected parameters for the generated BLR models.

3.5. Correlation and regression analysis
None of the 32 pre-hypoxia peak heights correlated significantly with the mean PASP over the last 30 min of the 90 min hypoxic exposure (table 4). One of the 32 hypoxia-induced changes in peak heights (peak at RT 69.321) correlated significantly with the mean PASP over the last 30 min of the 90 min hypoxic exposure (table 4). Forward stepwise linear regression analysis resulted in a significant fit to the data that used the hypoxia-induced change in peak height of this one correlated peak with a retention time of 69.32 min to predict the mean PASP over the last 30 min of the 90 min hypoxia exposure (figure 6).

Eleven of the 32 pre-hypoxia peak heights correlated significantly with the mean SaO2 over the last 30 min of the 90 min hypoxic exposure (table 4). Three of the 32 hypoxia-induced changes in peak height correlated significantly with the mean SaO2 over the last 30 min of the 90 min hypoxic exposure (table 4). Forward stepwise linear regression analysis resulted in a significant fit to the data that used heights of pre-hypoxia peaks with retention times of 76.55, 116.40 and 69.32 min and hypoxia-induced changes in peak heights calculated from peaks with retention times of 132.63, 77.45 and 116.40 min to predict the mean SaO2 over the last 30 of the 90 min hypoxia exposure (figures 7(a) and (b), respectively).
3.6. Chemical identification

Peak selection for chemical identification was based upon group differences in peak heights, and the results of BLR, correlation and stepwise linear regression analysis. Of the peaks selected using these criteria, 12 were identified with high confidence (table 5).

4. Discussion

To our knowledge, this is the first study that has examined hypoxic ventilatory responses, pulmonary artery systolic pressure responses to hypoxia and exercise and expired breath condensate analysis of VOCs in order to identify and compare markers of HAPE history. Our results provide evidence that volatile compounds found in EBC along with physiologic responses to hypoxia can be used to identify individuals who have a history of HAPE. The peak heights of 3 peaks in the pre-hypoxia gas chromatograms were significantly different between control and Hx HAPE groups. In addition, correlation and regression analysis demonstrated that there is a strong association between the level of certain VOCs in pre-hypoxia EBC and the magnitude of SaO2 response to acute normobaric hypoxia. The combined results of the direct comparison of pre-hypoxia GC peak heights, BLR and linear regression identifies 5 compounds that may be markers of HAPE history. Subsequent chemical identification points to the amount of benzyl alcohol and dimethylbenzaldehyde dimethyl in expired breath as markers of HAPE history.

Our study was consistent with previous research in that individuals with a history of HAPE have a blunted HVR (Hohenhaus et al 1995) (figure 1). The PASP response to supine exercise was not different between groups (figure 2), a finding that contrasts with previous studies employing the same protocol (Kawashima et al 1989, Eldridge et al 1996, Grunig et al 2000, Dehnert et al 2005). We attribute this discrepancy to the small sample size of our HAPE group, and the difficulty in obtaining an accurate tricuspid regurgitant signal at workloads over 75 W. Individuals with a history of HAPE also have an exaggerated HPV that could contribute to a greater increase in pulmonary artery pressures (Grunig et al 2000, Dehnert et al 2005). During hypoxic exposure, the pulmonary vasculature constricts in hypoxic regions in order to maintain alveolar ventilation and capillary blood flow matching. At high altitudes, this vasoconstriction becomes global rather than localized, and can cause pulmonary artery pressure to rise. In this study, PASP response during the 90 min of hypoxic exposure was significantly greater at 45, 60 and 75 min of hypoxia in the Hx HAPE group compared to the control group (figure 3(a)). In addition, the mean PASP response of the last 30 min of the 90 min hypoxic exposure was greater in the Hx HAPE group than the control group. When using these physiological variables as inputs for stepwise BLR, we developed a discriminatory model that contains HVR, PASP and SaO2 responses to hypoxia as predictive variables.

Two approaches were used to generate models to test whether information contained in the GC/MS data could be used to discriminate between subjects with and without a history of HAPE. Auto-regression/partial least square regression and stepwise BLR were used to analyze the whole chromatograms and individual peak data, respectively. While the AR/PLSR appears to produce a good separation of the data (figure 5), the separation accuracy was only 50.0 and 68.8% for the pre- and post-hypoxia chromatograms, respectively. These separation accuracy values are less than the value of 75.4% that we obtained when we compared age-matched controls to patients with idiopathic pulmonary arterial hypertension (IPAH) (Mansoor et al 2014) using the same analytical methods. This may be in part due to the relatively high number of peaks that were unique to the two groups in the IPAH study and the greater number of total subjects in that study (57 versus 16). Also, the observation that there were 1814 peaks that were identified in the current study that were present in 3 or less subjects and the relatively few peaks that appear to be discriminatory for HAPE history...
suggest that the signal to noise ratio is poor in the whole chromatograms suggesting that AR/PLSR analysis may be inappropriate for this data set. Applying stepwise BLR to the final 32 peaks present in at least 4 subjects in the pre- and post-hypoxia EBC samples resulted in separation accuracies of 100.0 to 87.5% (table 3). The stepwise BLR using the pre-hypoxia peaks resulted in a model that used the peak heights for peaks with retention times of 97.75 and 116.41 min. Interestingly, when stepwise BLR was applied to the whole data set containing pre- and post-hypoxia peak heights as well as the change in peak heights with hypoxia, a model containing the pre-hypoxia peak with a retention time of 97.75 min and the change in peak height of the peak with a retention of 116.41 was generated. These results suggest that dimethylbenzaldehyde (97.75 min) and N,N-dibutylformamide (116.41 min) are potential discriminatory markers for HAPE history. The results of the BLR analysis should be interpreted with caution, since, as with the AR/PLSR analysis, the number of subjects is small and it was therefore not possible to validate the models generated.

Correlation (table 4) and regression analysis (figures 6 and 7(a)) between the mean PASP and SaO₂ responses over the last 30 min of the hypoxia exposure and the pre- and post-hypoxia peak heights suggest a possible association between the peak identified as benzyl alcohol with a retention time of 69.32 min and the PASP and SaO₂ response. Interestingly, the peak identified as 1-methyl-4-(1-methylethenyl)-benzene with a retention time of 77.46 min was correlated with the SaO₂ response to hypoxia was also identified in controls and PAH patients in a previous study (Mansoor et al 2014). In addition, correlation and regression analysis between the mean SaO₂ response over the last 30 min of the hypoxia exposure and peak heights from pre- and post-hypoxia again highlights the predictive value of the amount of N,N-dibutyl-formamide in expired breath as a marker of hypoxia-induced SaO₂ response and HAPE history. It should be pointed out that the

### Table 3.
Results of separation analysis using AR/PLSR on whole chromatograms and binary logistic regression on individual peak height data.

| Input variables                             | Type of analysis | Selected variables | \( R^2 \) | Separation accuracy (%) |
|---------------------------------------------|-----------------|--------------------|----------|------------------------|
| Pre-hypoxia whole chromatogram             | AR/PLSR         | NA                 | 0.563    | 50                     |
| Post-hypoxia whole chromatogram            | AR/PLSR         | NA                 | 0.656    | 68.8                   |
| HVR, PASP and SaO₂ response to hypoxia     | sBLR            | HVR, PASP and SaO₂ response to hypoxia | 1.000    | 100                    |
| Pre-hypoxia peak heights                   | sBLR            | Peaks with RT of 97.75 and 116.41 min | 1.000    | 100                    |
| Post-hypoxia peak heights                  | sBLR            | Peak with RT of 117.49 min | 0.593    | 87.5                   |
| Hypoxia induced change in peak heights     | sBLR            | Peaks with RT of 42.06 and 82.81 min | 0.767    | 93.8                   |
| All variables                              | sBLR            | Pre-hypoxia peak with RT of 97.75 min and hypoxia-induced change in peak with RT of 116.41 min | 1.000    | 100                    |

HVR, hypoxic ventilator response; PASP, pulmonary artery systolic pressure; SaO₂, arterial oxygen saturation; AR/PLSR, auto regression/partial least square regression; sBLR, stepwise binary logistic regression; RT, retention time.

### Table 4.
Significant correlations (\( p \leq 0.05 \)) between peak heights or changes in peak heights during normobaric hypoxia and SaO₂ or PASP.

| Retention time (min) | Pre-hypoxia peak height | Absolute change in peak height w/hypoxia |
|----------------------|-------------------------|------------------------------------------|
|                      | SaO₂⁺                   | PASP⁺                                    |
|                      | SaO₂⁻                   | PASP⁻                                    |
| 65.266               | 0.541 (0.031)           | —                                        |
| 66.606               | 0.581 (0.018)           | —                                        |
| 69.321               | —                       | —                                        |
| 76.533               | 0.653 (0.006)           | —                                        |
| 77.458               | 0.592 (0.016)           | —                                        |
| 82.815               | 0.557 (0.025)           | —                                        |
| 96.550               | 0.549 (0.028)           | —                                        |
| 97.754               | 0.587 (0.017)           | —                                        |
| 111.426              | 0.629 (0.009)           | —                                        |
| 116.407              | 0.619 (0.011)           | —                                        |
| 117.488              | 0.525 (0.037)           | —                                        |
| 132.631              | 0.639 (0.008)           | —                                        |

SaO₂⁺ and PASP⁺ are averages from 60 to 90 min of normobaric hypoxia. SaO₂⁻, arterial oxygen saturation; PASP⁺, pulmonary artery systolic pressure.

Note: Pearson correlation coefficients (\( p \) value).
compounds found in the present work to discriminate the HAPE group may originate from various sources (de Lacy Costello et al. 2014). One of the detected biomarkers, benzyl alcohol, is a common environmental contaminant and may not be of endogenous origin. However, the rest of the detected biomarkers are not associated with environmental contaminants (Kwak and Preti 2011 #1520) and thus are not likely to be from extraneous sources. At the same time, several of these compounds are not normally associated with mammalian metabolism and thus their exact origin warrants some elucidation. Several of the compounds, such as dimethylbenzaldehyde may originate as a result of chemical reactions involving antioxidants. The number of compounds reported in table 5 can be formed enzymatically or non-enzymatically from both endogenous and exogenous antioxidant compounds. Many of the antioxidants that can lead to formation of compounds reported in table 5 are a part of a normal diet. The examples of these compounds are vitamin E, folic acid, as well as a variety of compounds such as flavonoids. The latter, although widely distributed in plants, are not produced endogenously by mammals. The flavonoids are quickly oxidized and excreted in the body and also result in concomitant production of uric acid, also an antioxidant. The N,N-dibutylformamide compound can be formed in radical reactions from urea or uric acid, or they may result from degradation/interconversion of larger molecules, while hydrocarbon compounds may result from oxidative cleavage of lipids (Amann and Smith 2013 #1073). Clearly, further studies are needed to establish mechanistic details of formation and biological basis for the detected biomarker compounds. The results presented here, however, are consistent with possible alteration of oxidative chemistry in HAPE subjects during hypoxia events. One of the future studies may be an evaluation of volatiles upon ingestion of isotope-labelled antioxidant compounds in order to evaluate whether the excreted volatiles originate from certain sources.

While the sample size was small, we were still able to obtain significant differences in HVR, PASP response to hypoxia and peak heights of selected VOCs in the EBC GC/MS data of our control and Hx HAPE groups. However, the small sample size restricted our ability to adequately validate the predictive models generated using AR/PLSR or BLR. Another limitation of this study was that the control group was selected from the general population, many of whom had never stayed the night at high altitude before. Given their unknown reactions to altitude, these subjects might have developed HAPE if they had ascended to high enough altitudes quickly. Indeed, two individuals in the control group had PASP at the end of the 90 min hypoxia exposure in excess of 40 mmHg. This limitation is not unique to this study. In fact all the cited studies (Hultgren et al. 1971, Kawashima et al. 1989, Matsuzawa et al. 1989, Hohenhaus et al. 1995, Eldridge et al. 1996, Grunig et al. 2000, Dehnert et al. 2005, Swenson and Bartsch 2012) that examine
physiologic end-points in HAPE ‘susceptible’ subjects compare individuals with a history of HAPE to individuals without a history of HAPE. In most of these studies the individuals without a history of HAPE have extensive experience at altitude, however, it should be recognized that this does not preclude them from developing HAPE in the future. This general limitation in HAPE ‘susceptibility’ studies leads to the following questions. Are the differences observed in HVR and pulmonary vascular responses to hypoxia and exercise in these studies indicative of HAPE susceptibility and were they present before the subjects developed HAPE? Alternatively, are these responses to hypoxia markers of HAPE history and indicative of a prolonged HAPE-induced alteration in ventilatory and pulmonary vascular responses to hypoxia?

5. Conclusion

Our findings demonstrate there is a difference in EBC chemical profiles between HAPE susceptible individuals and control subjects. These findings suggest that EBC analysis may provide a tool that can be used to discriminate an individual’s history of HAPE, as well as other physiological responses to hypoxia. Future studies with expanded group numbers need to be conducted that not only divide subjects based on HAPE history but also on the basis of their PASP response to hypoxia. This approach would not only identify markers of HAPE history, but could potentially identify markers of HAPE susceptibility in a naive population.

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Table 5. Chemical identification of peaks selected based on group differences and the results of binary logistic regression, correlation and stepwise linear regression analysis.

| Peak retention time (min) | Proposed chemical structure | Other possible chemical structures |
|--------------------------|-----------------------------|----------------------------------|
| 65.266                  | Heptane, 3,3,3-trimethyl      |                                  |
| 66.606*                 | Benzene, methyl (1-methyl)    |                                  |
| 69.321                  | Benzyl alcohol               |                                  |
| 76.553                  | Campholenol,6-               |                                  |
| 77.458*                 | Benzene, 1-methyl-4-(1-methyl) | Benzene, (2-methyl-1-propenyl)-Benzene, methyl(1-methylphenyl)- |
| 82.815*                 | Campholenaldehyde            |                                  |
| 96.550                  | Unsaturated hydrocarbon (gt10 carbons) | Benzaldehyde, 2,4-dimethyl-Benzaldehyde, 2,3-dimethyl-Benzaldehyde, 5,4-dimethyl |
| 97.754*                 | Benzaldehyde, 2,5-dimethyl-   |                                  |
| 111.426                 | Unsaturated hydrocarbon (approx 14 carbons) |                                |
| 116.407*                | Formamide, N,N-dibutyl-       |                                  |
| 117.488                 | 2-Isopropyl-5-methyl-1-heptanol |                                  |
| 132.631                 | 1H-3a,7-Methanoazulene,      |                                  |
|                         | 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl |                  |

*Peaks identified with ‘high confidence’, i.e. these structures were more likely to be the correct match than other potential candidate compounds as described in section 2.

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