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NOTE

Exhaled particle size and phospholipid composition of the respiratory tract lining fluid following moderate-intensity exercise in sub-zero conditions

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Abstract

Exercise-induced bronchoconstriction (EIB) commonly develops after exercise in cold, dry environments, but the effects of acute exercise in the cold on airway physiology remain incompletely understood. Exhaled particle (PEx) analysis offers a novel, non-invasive approach to assess respiratory tract lining fluid (RTLF) composition and may provide mechanistic insights into airway responses before clinically detectable changes occur. This study investigated the PEx response to moderate-intensity exercise in sub-zero conditions among healthy atopic and non-atopic individuals. Eighteen participants (14 male, 29 $\pm$ 6 years) performed two moderate-intensity exercise trials (30 and 90 min duration) in a climate chamber at -15 °C. PEx samples were collected using the PExA® method, before and 30 min after exercise to assess particle mass and count across eight size bins (0.4–5 μm) and for subsequent lipidomic analysis. Exercise induced a significant increase in smaller PEx (0.4–0.7 μm; $p < 0.01$). Three lipid species were altered after 30 min exercise, and 11 after 90 min exercise. Phosphatidylethanolamine PE(16:1_18:0) was the only lipid that consistently increased across both exercise durations (30 min: $p = 0.023$; 90 min: $p = 0.044$; $g = 0.97$). Three specific lipid species showed differential exercise responses between atopic and non-atopic participants, including an oxidised phosphatidylcholine species PC(16:0_9:0;O) ($p = 0.006$, $q = 0.59$, $g = 0.86$). These results provide preliminary insight into airway surfactant responses to exercise in a cold climate and contribute to the growing understanding of how the PExA® method can be applied as a non-invasive tool for respiratory health assessment in an acute setting.

1. Background

The combination of exercise and exposure to airway irritants is recognised as an occupational health risk to athletes [1–3] and the general population exposed through work or leisure activities [4, 5]. Athletes in high-performance endurance sports, such as running, swimming and cross-country skiing, which demand sustained high ventilation rates, have a high prevalence of asthma [6–9]. However, the occurrence of respiratory symptoms, acute reductions in lung function, and airway inflammatory responses is particularly high in settings where athletes are exposed to swimming pool chemicals [10], environmental air pollution [11], pollen [12], and cold, dry air [12, 13] during training. These irritants have been identified as triggers for exercise-induced bronchoconstriction (EIB) and are likely involved in

the initial development of lower airway dysfunction among athletes [12, 14–16]. While lower airway dysfunction, an operational definition encompassing asthma, EIB and airway hyperresponsiveness [8], has been well-recognised in winter endurance athletes, such as cross-country skiers, for over 30 years [17–19], its prevalence remains relatively unchanged over time [8], and we still lack complete understanding of the airway pathophysiology induced by exercise in sub-zero conditions. Briefly, inhalation of cold, dry air causes desiccation of the airway surface liquid, creating a hyperosmolar environment that leads to shrinkage of surrounding cells and the release of inflammatory mediators such as histamine, leukotrienes and prostaglandins. The resulting smooth muscle contraction, alongside reactive hyperaemia-induced oedema upon rewarming, can elicit EIB [20]. However, we have limited evidence to map the cascade of exposures and events leading over time to development of long-term lower airway dysfunction in a prospective manner.

Regular exercise in a cold environment involving high rates of ventilation may over time initiate a cycle of epithelial injury and repair that contributes to the development of airway hyperresponsiveness [20]. A handful of cross-sectional and longitudinal studies have used samples from bronchial biopsies using bronchoscopy and induced sputum to characterise the immune cell and inflammatory profile of the airways following chronic exposure to cold environments [21–24]. However, bronchoscopy is invasive and induced sputum is highly dependent on subject cooperation and technique, risk of contamination from upper airways, and variable reproducibility. Several studies have used blood and urine biomarkers to ascertain whether exercise and/or environmental exposures affect airway epithelial integrity [25–30]. Nevertheless, the ability to identify, validate and assess a larger panel of biomarkers of airway integrity directly from the respiratory tract before and after acute exercise bouts, using minimally invasive methods, could ultimately lend itself to non-invasive monitoring of airway exposure in clinical and field settings.

The respiratory tract lining fluid (RTLF) covering the airway epithelium constitutes a central line of defence against environmental insults. Analysis of the composition of the RTLF can be evaluated using a novel, non-invasive method termed PExA®, to collect exhaled particles (PEx) [31]. PEx from the RTLF, generated by opening and closing of the airways, have been shown to reflect peripheral and small airway inflammation [32, 33] and be affected by environmental triggers, such as smoking [34], allergens [32], and occupational exposures [35]. To date, the method has not yet been employed to assess airway responses to acute exercise in cold environments, a condition known to induce hyperpnoea leading to airway cooling and drying, that contributes to epithelial injury [36]. Analysis of RTLF composition via PExA® therefore offers an opportunity to explore early perturbations to the RTLF after exercise before clinically detectable changes in lung function occur. Pulmonary surfactant, composed primarily of phospholipids, helps to maintain alveolar stability and optimal gas exchange [37, 38] as well as serving as a protective barrier against mechanical stress and environmental insults for the airway epithelium [37, 39]. Notably, alterations in airway surfactant composition have been associated with reduced lung function in asthma [40], raising the possibility that similar changes, potentially triggered by exercise hyperpnoea in the cold, could increase airway vulnerability to injury.

Existing data suggest that exercise performed at high-intensity elicits greater bronchoconstriction and airway epithelial damage than low-intensity training [19]. However, the majority of the training that skiers perform is in the low-intensity domain [41–43], with prolonged sessions >1.5 h making up a substantial part of skiers' total training volume [44]. Single bouts of prolonged, low-intensity exercise have been associated with systemic immune disturbances [45, 46], which could possibly impair the ability of immune cells to promptly engage in epithelial repair post-exercise. While the repeated damage-repair cycle likely elicited by frequent endurance training in sub-zero environments may offer an explanation as to why cross-country skiing has been highlighted as a risk factor for development of asthma [2], to our knowledge no studies have investigated effects of prolonged endurance exercise in sub-zero conditions on the composition of the RTLF.

The purpose of this exploratory study was to investigate whether particles in exhaled air can be used to monitor the effects of exercise in cold air on the airways, and to characterise the acute impact of hyperpnoea in subzero air on the molecular integrity of the RTLF. A secondary aim was to investigate whether the particle and lipid profile of PEx samples was affected by exercise duration and participants' atopic status.

2. Methods

2.1. Participants

Eighteen participants (♂ = 14, ♀ = 4, age: 29 ± 6 years; VO_2max: 61.6 ± 8.6 ml kg^{-1} min^{-1}; mean ± SD) provided written, informed consent to participate in the study. Participants were currently engaged in regular endurance training but had never competed in winter endurance sports at elite level

(Tier 1–3 according to McKay *et al* [47]). Atopic status was determined using the Allergy Questionnaire for Athletes (AQUA©) [48]. Atopy was defined as a questionnaire score $\geqslant$5. A detailed description of participants' anthropometric characteristics and training background has been previously reported [49]. The study was approved by the Swedish Ethical Review Authority (2020–05205). All participants provided full written, informed consent to participate and the study was conducted in accordance with the 2013 version Declaration of Helsinki, but was retrospectively registered (ISRCTN13977758).

2.2. Study design

The study employed a 2 $\times$ 2 randomised crossover design whereby participants performed a preliminary test to determine $\dot{V}O_2$max at room temperature, followed by two treadmill running exercise trials, of 30 and 90 min duration, separated by approximately one week, in an environmental chamber at -15 °C and $\sim$67% relative humidity. Two exercise durations were selected to reflect ecologically relevant cold-air exposure—a brief exposure that may occur during recreational physical activity (30 min) and an exposure representative of deliberate endurance training (90 min). The exercise intensity was selected to be achievable for participants to sustain for 90 min and reflective of the low-to-moderate intensity domain that constitutes the majority of winter endurance athletes' training [44]. Treadmill speed during the exercise trials was 10.0 $\pm$ 1.6 km h^{-1}. PEx were collected at two time points, before and 30 min after exercise. An individualised, prescribed treadmill speed for the main exercise trials was determined by interpolation of data from the submaximal stages of a preliminary submaximal and maximal exercise test to determine a speed that would elicit approximately 60% $\dot{V}O_2$max during the subsequent visits. A detailed methodology of the pre-test and chamber exercise trials, as well as physiological responses to exercise, lung function results and blood biomarkers measured during the study has been previously published elsewhere [49].

2.3. Exhaled particle collection

The composition of the RTLF can be assessed using a novel and non-invasive method to collect and analyse particles in exhaled air using a patented instrument (PExA®, patent P17584SE00). The instrument can collect and discriminate larger (0.4–5 μm) particles from the RTLF generated by opening and closing of the small airways [35, 50], herein referred to as PEx. The PExA® instrument can be used to obtain a particle mass and count, both in total and across different particle size domains, while collection and storage of the collected particles enables subsequent assessment of airway surfactant phospholipid composition.

During PEx collection, participants performed multiple repetitions of a specific breathing manoeuvre, whereby the participant exhaled to achieve residual volume, then held their breath for 5 s. This was then followed by a steady inhalation to vital capacity before a slow exhalation (sVC) into the device. Inhalation of particle-free filtered room air was achieved through a two-way non-rebreathing valve (1420, Hans Rudolph, Shawnee, KS, USA), followed by immediate exhalation into the device. The breathing manoeuvre was repeated at self-regulated intervals. Due to the limited time window for particle collection and the need to collect particles within a similar time window for all participants, particle collection was terminated as soon as the participant reached one of the following end points: 120 ng particle mass had been collected (sufficient particles obtained), after 15–16 exhalations (to prevent fatigue that could affect later tests), or after approximately 10 min (due to time constraints and to match the post-exercise time point within- and between participants; i.e. 30–40 min post-exercise).

The concentration of particles was measured in eight size bins between approximately 0.4–5 μm in diameter by optical particle counter in the PExA® instrument (PExA AB, Gothenburg), as described by Almstrand *et al* [50]. The PExA® computer software calculates the sampled particle mass online and displays collected mass to the participant. Particles are collected by the inertia principle using a cascade impactor. Particles are impacted on a thin hydrophilic polytetrafluoroethylene membrane mounted on the impactor collection plate. When sampling had finished, the sampling membrane was taken out of the impactor, divided in two using a clean scalpel, and stored in a microcentrifuge tube at -80 °C until later analysis.

2.4. Lipidomics analysis of PEx samples

A detailed procedure for analysing surfactant lipids from PEx samples of small airways has been described previously [51]. The lipids included in the method were selected during a method development phase preceding this study. Lipid panel selection was based on screening pooled PEx samples using Waters LipidQuan Quanpedia lipid MRM database (Milford, MA, USA). In addition to the targeted screening, untargeted screening was conducted using lipid class specific fragmentation scanning experiments, such as neutral loss scanning and precursor ion scanning. The most abundant lipids in each class were selected for confirmation and further validation (selectivity, precision, linearity) before inclusion into the lipid panel used in the present study.

In brief, samples were placed in the insert of centrifugal filter tubes (Millipore UFC30LG25). To the sampling membrane 10 μl of isotope labelled lipid

standard was added and allowed to dry with the sample for 10 min (Avanti EquiSPLASH SKU:330731 and Avanti GlcCer(d18:1(d5)/18:0) SKU:860638). Lipids were extracted in a thermomixer using 225 μl of isopropanol. The eluate was recovered from the insert to the microtube bottom by centrifugation. The extraction was then immediately repeated with methanol for improved recovery of both the polar and the neutral lipids. The methanol/isopropanol eluate in the microtube was transferred into LC vials (Waters Total Recovery vial SKU: 186002805) and stored at -20 °C before analysis. Before analysis, the solvent in the LC-vial was evaporated under a stream of nitrogen and the remaining lipid film was solubilised in 100 μl of solvent consisting of acetonitrile and isopropanol in a ratio of 2:1. The extracted lipids were analysed with a targeted LC-MSMS method using the Waters ACQUITY UPLC I-Class chromatography system coupled to an electrospray ionization source on a Waters Xevo TQ-XS triple quadrupole mass spectrometer detector (Milford, MA, USA). Reverse phase chromatography was used for separation of PC lipids based on fatty acyl groups. All other lipids were analysed using HILIC chromatography that separates the lipid classes.

Quantification was based on the peak area of the analyte divided by the peak area of the internal standard, the response factor. The response factor was multiplied with the mol of internal standard spiked to the sample. One internal standard per lipid class was used. The results of the lipid analyses were calculated as relative amount [51]:

$$\left(\text{mol\%} = 100 \times \frac{\text{species\_mol}}{\text{lipid\_sum\_mol}}\right).$$

Type II isotope correction factors were calculated with the online correction tool LICAR for the lipid classes PG,PE and PI, as described by Gao *et al* [52]. When isomers were found as two distinct peaks then the first peak was annotated with an '_A' and the second with a '_B'. For example: LPC(16:1)_A and LPC(16:1)_B corresponds to the SN1 and SN2 position of the 16:1 fatty acyl group on the glycerol backbone.

2.5. Data analysis

The PExA® instrument provided data on total particle count and mass, as well as the distribution of particle count and mass across eight particle size bins. These data were normalised relative to the total volume of exhaled air during the collection. Three samples encountered technical errors during collection and were missing from analysis, one further sample was excluded from analysis due to suspected contamination. Data were analysed in GraphPad Prism (version 10.6, GraphPad Software, Boston, MA, USA).

First, total particle count and mass were analysed using linear mixed models with fixed effects for trial, timepoint and their interaction. Subsequently, the proportion of particles within each particle size domain was analysed using two models: first, with fixed effects for trial, timepoint and particle size, including their interactions, and subsequently, with fixed effects for timepoint and particle size only. Fisher's LSD (for 2×2 analyses) or Bonferroni post-hoc tests were used to follow up significant interactions.

Lipidomics data were reported as mol% of total sample lipid content. Lipids with $<$20% missing values were retained; remaining missing values were imputed using the lowest detected mol% divided by $\sqrt{2}$. Samples with a total collected mass of $<$10% of the target ($<$6 ng) were excluded ($n = 2$). Thus, analysis of lipid composition was ultimately performed on data from 66 samples.

For baseline analyses, the mean lipid mol% from each participant's baseline samples were calculated. For each trial, the change in mean mol% for each lipid from pre to post exercise was quantified using the $\log_2$ logit difference, calculated as:

$$\log_2\left(\frac{\frac{\text{mol\%}}{100}_{\text{post}}}{1-\frac{\text{mol\%}}{100}_{\text{post}}}\right) - \log_2\left(\frac{\frac{\text{mol\%}}{100}_{\text{pre}}}{1-\frac{\text{mol\%}}{100}_{\text{pre}}}\right).$$

Lipid responses to each individual exercise trial were analysed using paired t-tests; a prerequisite was thus that samples for both pre- and post- time points within a trial were available (30 min: $n = 14$; 90 min: $n = 17$). To meet the assumptions of parametric testing, lipid mol% data were arcsine transformed prior to statistical analysis, as this transformation stabilises variance and improves normality for proportion data bounded between 0 and 1. Results were visualised against the $\log_2$ logit difference using volcano plots in Prism. The $\log_2$ logit difference was chosen to quantify the magnitude and direction of change from pre- to post-exercise, offering an interpretable magnitude of change for visualisation, as it linearises proportion changes near 0 and 1 and facilitates comparison across trials. Differences in lipid mol% between atopic and non-atopic individuals at baseline, and $\log_2$logit responses to exercise, were analysed using independent t-tests. We applied a significance threshold of $p < 0.05$ for reporting biomarker responses of interest. Raw p values and FDR-corrected q values (Benjamini–Hochberg, $Q = 10\%$) are presented in parallel, but given no q values reached the threshold to be retained as true discoveries, p values were used to best describe changes in the data, but should be interpreted with caution. Hedge's g effect sizes are reported for pairwise comparisons to aid interpretation. Finally, principal component analysis (PCA) was performed to investigate potential clustering of lipids measured pre and post 30 and 90 min exercise, and

to assess differences in the lipid response to exercise based on atopic status.

3. Results

3.1. Effects of exercise on exhaled particle mass and count

The mean (± SD) number of exhalations during sample collection was 10 ± 2, although the obtained particle mass from each collection varied substantially between samples (82 ± 55 ng). Due to technical errors, particle data were not obtained at three collection time points.

Particle mass and count, normalised to the total exhaled air volume during sample collection, are shown in figure 1. The change in particle count from pre- to post-exercise differed significantly between trials ($F(1, 11) = 5.56$, $p = 0.04$). Post hoc analyses revealed a decrease in particle mass and count post-exercise in the 90 min trial, attributable to higher particle counts at baseline in the 90 min trial compared to both post-exercise and before the 30 min exercise trial (figure 1(A)). For particle mass, there was a main effect of trial, whereby exercise decreased particle mass ($F(1, 17) = 7.96$, $p = 0.012$).

Analysis of the response to exercise for particles across different size domains revealed a significant interaction between timepoint and particle size (PEx count: $F(7, 136) = 6.379$, $p < 0.0001$; PEx mass: $F(7, 136) = 12.81$, $p < 0.0001$). Follow-up analysis of these effects indicated a greater proportional count and mass of smaller particles (0.41–0.70 μm) post-exercise, and a lower proportional mass of larger (2.36–4.55 μm) particles post-exercise, as well as a lower count of particles sized between 0.70–0.92 μm post-exercise (figure 2).

3.2. Effects of exercise on exhaled particles assessed using lipidomics

In total, 93 lipid species were detected and retained for analysis. Phospholipid composition in all samples was dominated by PC(16:0/16:0) which made up more than 50% of the total lipids at all four time points (supplementary figure 1). In general, the relative proportions of the major lipids showed minimal change across the four sample time points.

Analysis of lipid responses to individual trials revealed three lipids that significantly differed ($p < 0.05$) from pre- to post-exercise in the 30 min trial (figure 3), and eleven in the 90 min trial (figure 4). The only lipid consistently altered across both trials was PE(16:1_18:0), which significantly increased following exercise (30 min: $p = 0.023$, $q = 0.865$; 90 min: $p = 0.044$, $q = 0.38$; combined time effect, $g = 0.97$).

3.3. Effect of atopic status on the PEx and lipid profile before and after exercise in a sub-zero environment

Based on the pre-defined AQUA score cut-off, 10 participants were classified as atopic (56%, 7 men, 3 women) and eight as non-atopic. There were no significant differences between the mean baseline PEx count or mass between the atopic and non-atopic groups (supplementary figure 2). Similarly, there were no significant differences in exhaled lipid composition at baseline between the two groups.

Analysis of the mean PEx response to exercise also showed no significant differences between the atopic and non-atopic groups (supplementary figures 2(C) and (D)). In addition, no differences in the distribution of PEx mass or count over the particle size domains were found between atopic and non-atopic participants (supplementary figure 3).

Three lipids were identified through volcano plots as potentially different in their response to exercise between the atopic and non-atopic groups (figure 5(A)): PG(18:0_20:4) ($p = 0.048$, $q = 0.92$, $g = 1.01$; figure 5(B)), PC(18:1_18:2) ($p = 0.026$, $q = 0.92$, $g = 0.99$, figure 5(C)) and PC(16:0_9;O), ($p = 0.006$, $q = 0.59$, $g = 0.86$, figure 5(D)). However, the distribution of particle mass across particle size bins was not affected by atopy (i.e. no significant main effect of atopy, nor atopy x timepoint interaction; supplementary figure 3).

3.4. Multivariate analysis

Analysis with PCA showed no clear separation between sample time points or trials (supplementary figure 4(a)), nor the difference in the response to exercise between atopic and non-atopic individuals (supplementary figure 4(b)). The lack of separation between groups and time-points in the PCA analysis precluded further analysis with supervised methods such as orthoPLS-DA to avoid overfitting [53].

4. Discussion

The present study investigated exhaled particle responses to moderate-intensity exercise in sub-zero conditions among healthy non-atopic and atopic participants, representing the first application of the PExA® method in an acute exercise setting. The main findings were that (1) a greater proportion of smaller particles were obtained after exercise (2) a higher number of lipids (eleven) were altered after 90 min exercise versus 30 min exercise (three), accompanied by a greater reduction in PEx count post-exercise after 90 min exercise (3) there was no significant difference in the composition of the obtained PEx mass and count between atopic and non-atopic participants at baseline nor in response to exercise, but

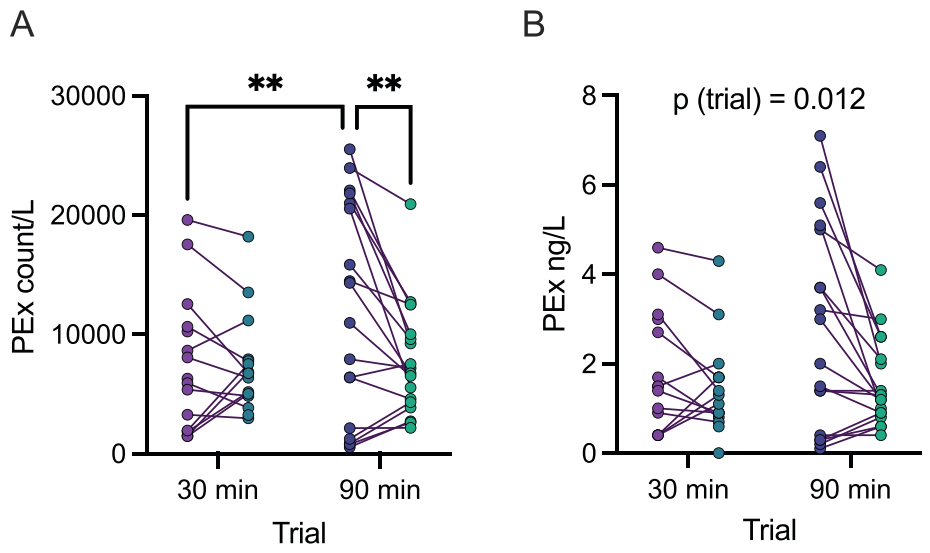

Figure 1. PEx count (A) and mass (B) pre and post 30 and 90 min exercise in −15 °C. Difference between time points, **, $p < 0.01$.

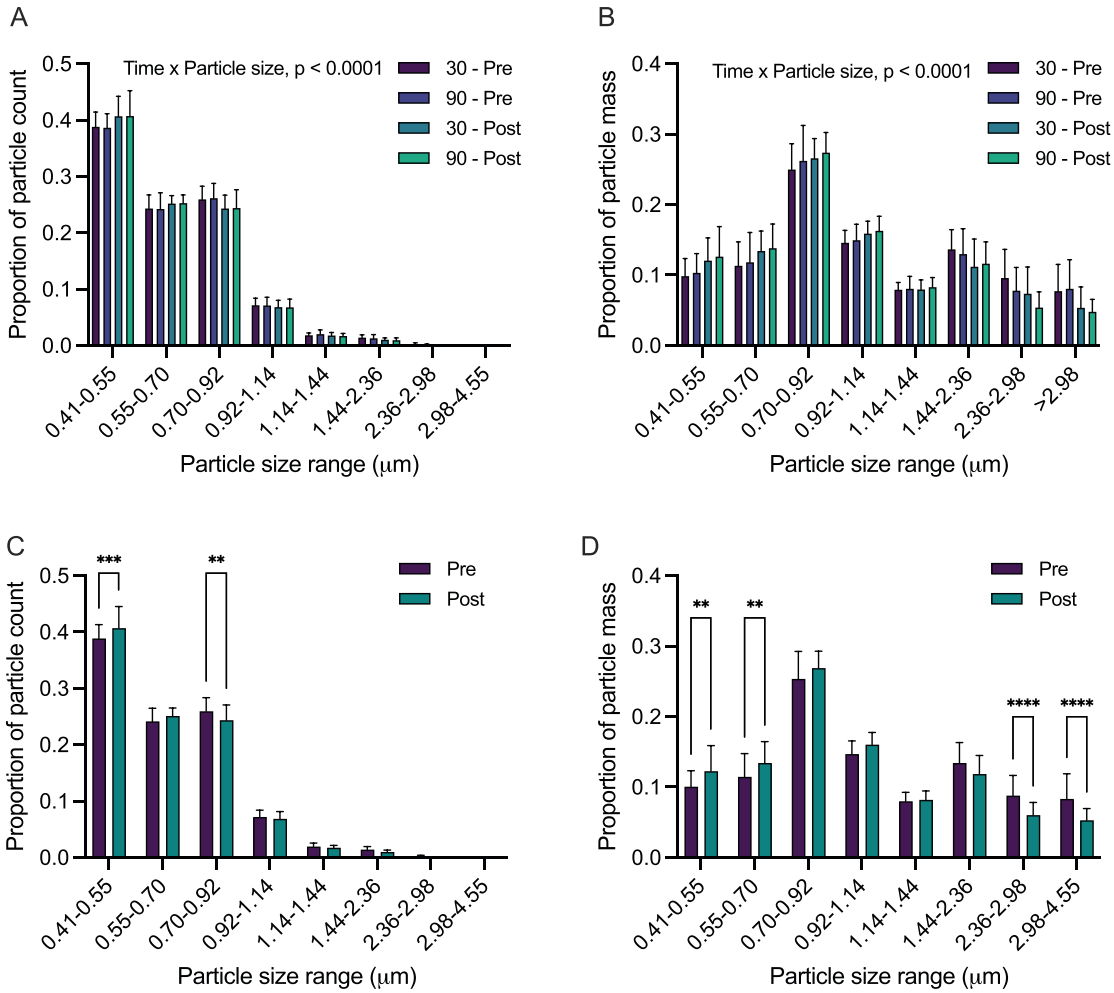

Figure 2. Particle count and mass within specific particle size domains pre and post 30 and 90 min exercise in −15 °C (Mean + SD). (A) Proportion of total particle count represented by particles in different size domains, across all four measurement time points. Significant post-hoc differences within size domains between timepoints are shown in panel (C). (B) Proportion of total particle mass represented by particles in different size domains, across all four timepoints. Significant post-hoc differences within size domains between timepoints are shown in panel (D). *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

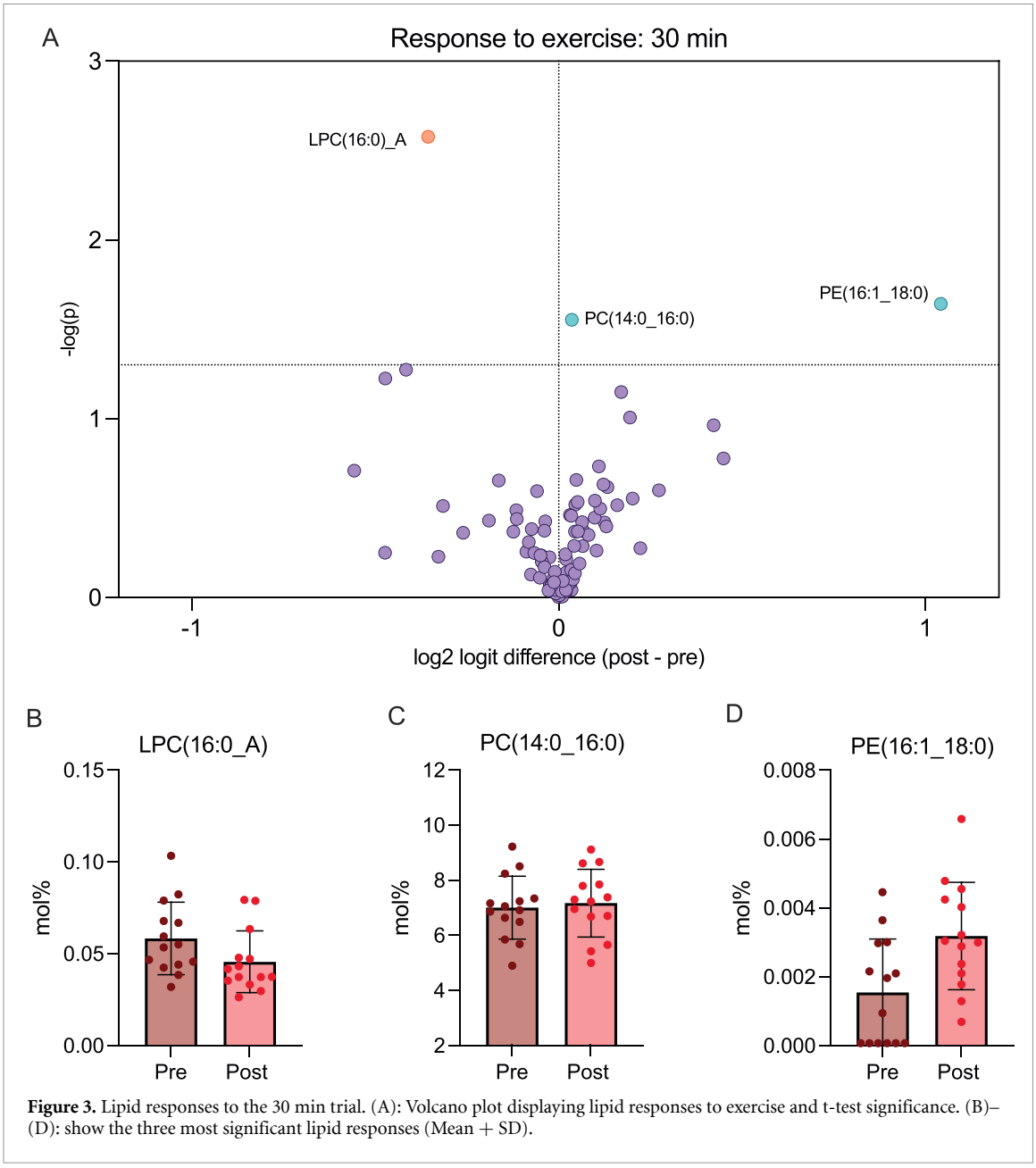

Figure 3. Lipid responses to the 30 min trial. (A): Volcano plot displaying lipid responses to exercise and t-test significance. (B)–(D): show the three most significant lipid responses (Mean + SD).

(4) three lipids showed differing exercise responses between non-atopic and atopic participants.

The consistent shift toward smaller particles (0.5–1 μm) following exercise across both trials represents a novel finding, suggesting that the physical properties of particle generation may have been altered by exercise in a subzero environment. The reduction in particle count and mass after exercise likely reflects subtle alterations in airway surfactant properties. While not causing functional impairment in this acute setting, this observation may provide mechanistic insights into the pathogenesis of EIB with repeated exposure to exercise in cold air. While no participants in the present study had clinically relevant changes in FEV1 post-exercise [49], previous observations have shown an association between decreased PEx mass and increased asthma severity [54], suggesting that PEx measurements may be sensitive to early perturbations in respiratory tract integrity even before clinically detectable lung function changes occur. The increased ventilation of exercise in combination with the cold, dry environment leads to cooling and drying of the airway epithelium, which could affect mucosal hydration and viscosity, surface tension and airway reopening dynamics [55]. The volume of the RTLF in the lower airways has been modelled to be under 1 ml, and thus susceptible to water loss and resultant osmotic stress [56], especially during high ventilation exercise in a cold, dry environment. It has been hypothesised that shear stress on the dehydrated airway epithelium can cause airway epithelial damage, leading to inflammation, release of mediators, increased epithelial permeability and plasma leakage into the airways, which over time can contribute

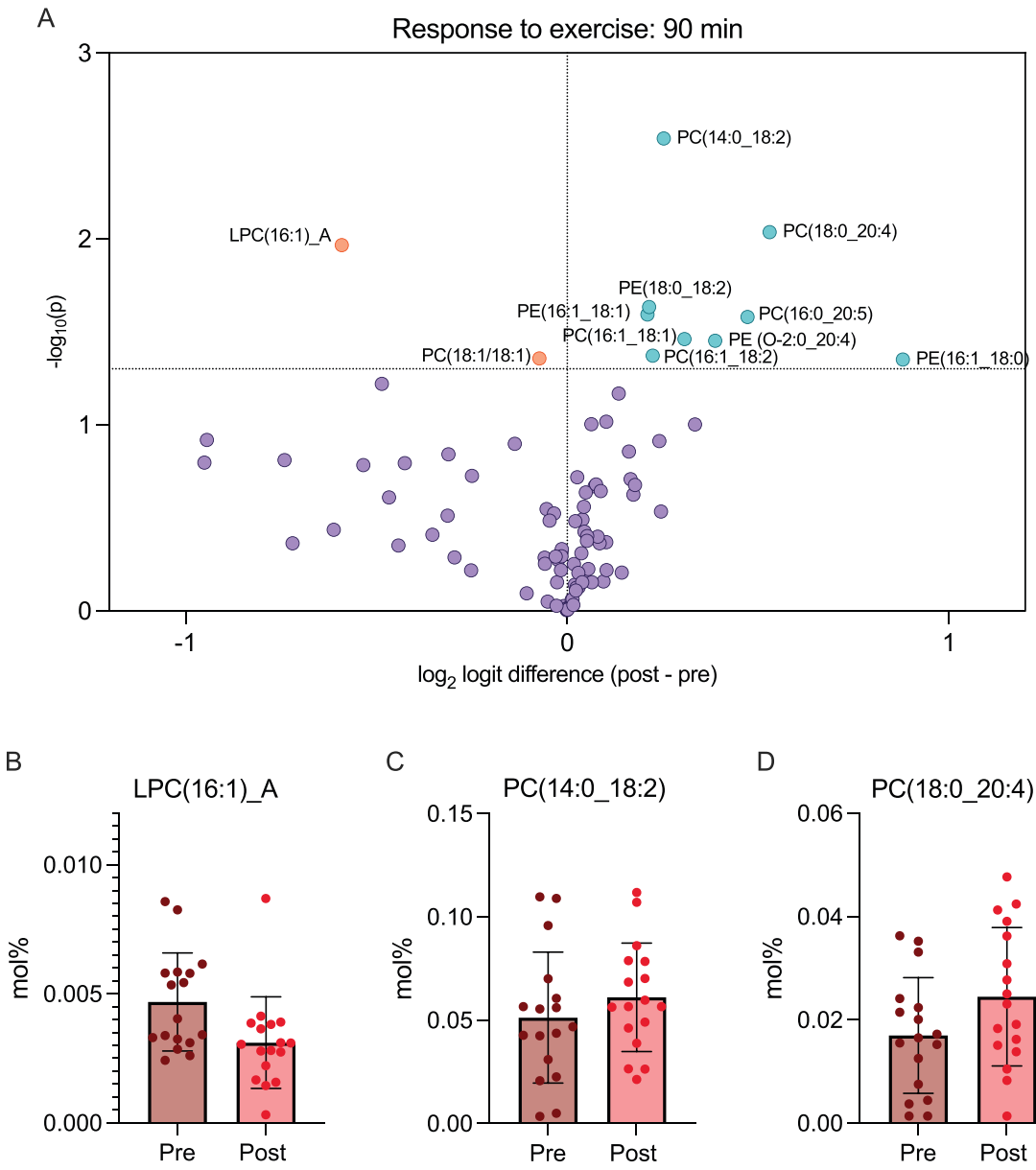

Figure 4. Lipid responses to the 90 min trial. (A): Volcano plot displaying lipid responses to exercise and t-test significance, (B)–(D): show the three most significant lipid responses (Mean + SD).

to airway remodelling and the development of airway hyperresponsiveness characteristic of EIB [36]. The impact of prolonged exercise in the cold on exhaled particle generation has not previously been explored, but we can speculate that drying may reduce the thickness of the RTLF during exercise, potentially leading to smaller particles being generated during airway reopening, whereas cooling and drying may also alter airway compliance and surface tension, that could also influence the mechanics of particle formation.

The responses of the detected lipid species to exercise, while modest, may also offer some insight into the RTLF response to exercise in cold air. The phosphatidylcholine PC(16:0/16:0) (dipalmitoyl phosphatidylcholine or DPPC) was the most abundant lipid across all samples at all time points, representing just over 50% of the total lipids, consistent with previous findings using the PEx method [33]. Only one lipid species, PE(16:1_18:0), showed a consistent increase after exercise across both trials. PE species are highly enriched in mitochondrial inner membranes [57], and the observed increase in PE(16:1_18:0) may indicate enhanced membrane biosynthesis or remodelling to support increased metabolic demands during and after exercise. In contrast, two lysophosphatidylcholine (LPC) species decreased post-exercise. LPCs are generated from phosphatidylcholines through phospholipase A2 (PLA2)-mediated hydrolysis and can impair

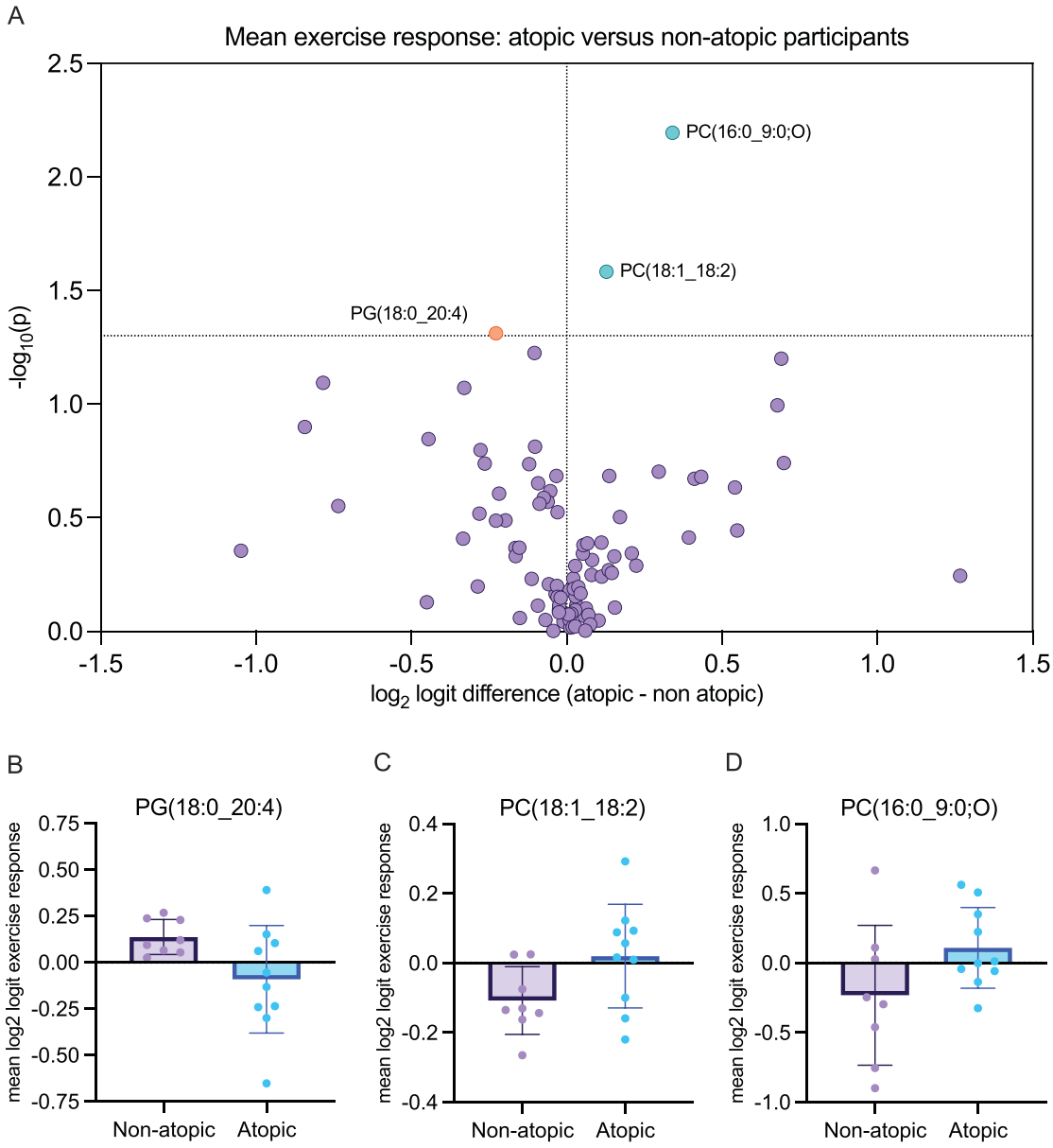

Figure 5. Mean lipid responses to exercise in non-atopic and atopic participants. (A): volcano plot showing significant lipids identified using unpaired *t*-tests (B)–(D). Visualisation of the mean (+ SD) exercise response for lipids that significantly differed between atopic and non-atopic participants.

surfactant function when elevated [58]. The observed decrease in LPC levels suggests reduced phospholipase activity and/or active reacylation of LPCs back to intact phospholipids during the recovery period, rather than ongoing epithelial disruption. The 30 min post-exercise sampling timepoint may therefore have captured the resolution phase following any transient perturbations during exercise. Atopic individuals showed a greater increase in an oxidised PC species, PC(16:0_9:0;O), potentially reflecting heightened oxidative stress, as previously reported in inflamed tissues [51]. We previously reported that atopic individuals experienced greater intensity of respiratory symptoms after exercise [49], so it is possible that their airways had a greater propensity to epithelial disruption or inflammation. As research into lipid regulation of airway integrity is still emerging, it remains uncertain whether the responses we observed may reflect a propensity to adapt to higher rates of ventilation, cold exposure, or cellular stress and the specific functional significance of the identified species in surfactant homeostasis requires further investigation.

Despite these changes in specific species, the overall lipid profile remained relatively stable at all measurement timepoints, and there were no significant shifts in lipid classes post-exercise. The overall greater number of lipid species that were altered ($p < 0.05$) after 90 min exercise versus 30 min exercise may indicate a dose-response relationship between exposure of the airways to acute epithelial stress and acute changes in RTLF composition. Taken together, the

reported changes in particle characteristics and lipid composition of PEx samples suggest that even a single bout of prolonged exercise in a subzero environment can perturb the RTLF. The overall stability of most lipid species and classes nevertheless suggests that the airway surfactant system is relatively robust and likely recovers quickly after environmental stress.

To our knowledge, this study was the first time that the PExA® method has been used to collect samples before and after exercise, and thus has value as a proof-of-concept study. Comparisons with studies that have used other breath sampling methodologies can provide important context for our findings. A recent proof-of-concept study using exhaled breath condensate also reported no change in breath biomarkers (8-isoprostane and pentraxin-3) after moderate-intensity exercise in healthy participants [59], with the authors suggesting that more extreme exercise intensities or co-introduction of environmental stressors would likely be needed to observe detectable biomarker changes. Similarly, previous findings from this project showed that prolonged (i.e. 90 min) moderate-intensity exercise did not elicit decreased lung function (measured using dynamic spirometry) nor substantial airway epithelial damage (assessed via plasma club cell Clara protein 16, CC16, concentrations), compared to 30 min exercise at −15 °C [49]. These findings suggest that the moderate-intensity exercise employed in the present study, even in sub-zero air, does not constitute a sufficiently potent stimulus to stress the airway mucosa and RTLF composition in healthy individuals. Whether a more potent stimulus (higher intensity) or susceptible population (i.e. individuals with EIB) would elicit greater perturbations in RTLF composition remains a question for future research.

Several limitations should be acknowledged when interpreting the findings from the present study. The elevated baseline particle count before the 90 min trial introduces complexities in interpretation of the findings; apparent post-exercise alterations in PEx may be attributable to this difference. The reason for the elevated baseline could not be attributed to trial order, number of breathing manoeuvres, or any other known variable. However, previously the within-subject coefficient of variation for PEx count has been reported at 27%, but in the present study was 76%, suggesting that day-to-day variability may have contributed to this difference [60]. Additionally, the shorter sampling window used in this study compared to previous work (10 min was the maximum allocated time in the study protocol for PEx collection to permit continuation to other time-sensitive measures) and the wide range in collected sample mass may have contributed to variation in sample composition. The study did not utilise a seated rest control trial, that may have highlighted any possible effects of time of day and/or other laboratory measurements (e.g. spirometry manoeuvres) on the PEx collection. Similarly, it was beyond the scope of this project to separate effects of exercise and cold, which would have required a more complex design including room-temperature exercise trials. Given the exploratory nature and multivariate dataset, as well as the proportion-shape of the mol% data, the relatively small sample size [61], and the lack of discoveries retained after FDR-correction, we should be aware that lipid species responses where $p < 0.05$ but $q > 0.05$ require further investigation and replication to verify our observations. Finally, since this was the first study to incorporate PEx measurements after physical activity, we highlight that no studies have yet been performed to determine the optimal post-exercise timepoint to detect possible alterations in the composition of the RTLF; this remains a question for future research.

5. Conclusion

The present study represents the first, to our knowledge, application of the PExA® method to exercise physiology research, and provides important preliminary data regarding the exhaled particle response to exercise under environmental stress. Our findings demonstrate that moderate-intensity exercise in sub-zero conditions induces subtle but measurable changes in exhaled particle composition, including a reduction in particle size and shifts in selected lipid species, such as a proportional increase in PE(16:1_18:0). These changes may reflect early markers of airway surface dehydration or surfactant remodelling, and may be used as a basis to inform future hypotheses and follow-up studies. The overall stability of the PEx composition nevertheless suggests the airway surfactant is relatively robust and recovers quickly after environmental stress, in agreement with our earlier findings of minimal changes in lung function or airway epithelial damage from the study protocol. Thus, our data provide preliminary insight into airway responses to exercise in a cold climate and contribute to the growing understanding of how the PExA® method can be applied as a non-invasive tool for respiratory health assessment in an acute setting.

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Data availability statement

The data cannot be made publicly available upon publication because they contain sensitive personal information. The data that support the findings of this study are available upon reasonable request from the authors.

Supplementary Figures available at: https://doi.org/10.1088/1752-7163/ae7fb0/data1.

Conflict of interest

A-CO is a board member and shareholder of PExA AB, the company that produces and sells the PExA instrument used in the present study. PL is a shareholder of PExA AB. All other authors have no conflicts of interest to declare.

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