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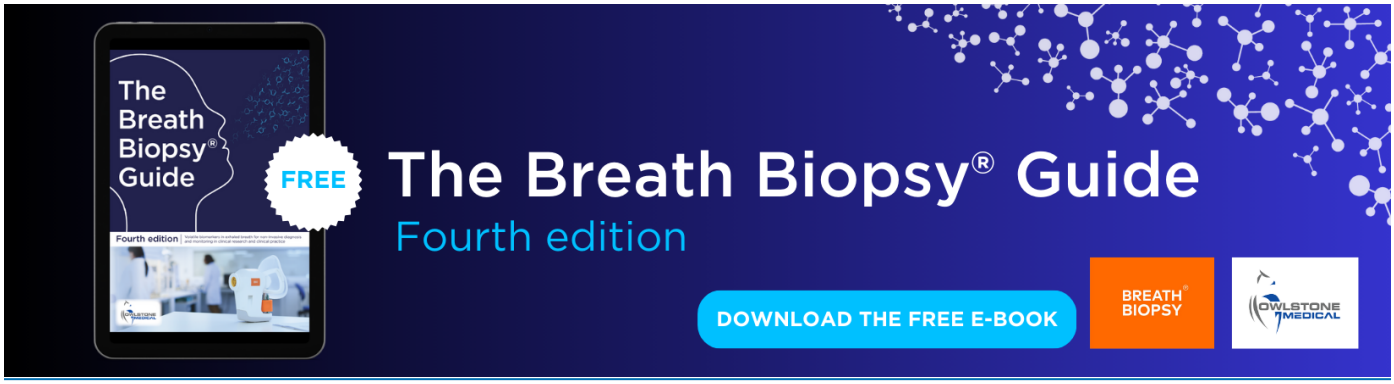

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PAPER

Metabolic insights at the finish line: deciphering physiological changes in ultramarathon runners through breath VOC analysis

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Abstract

Exhaustive exercise can induce unique physiological responses in the lungs and other parts of the human body. The volatile organic compounds (VOCs) in exhaled breath are ideal for studying the effects of exhaustive exercise on the lungs due to the proximity of the breath matrix to the respiratory tract. As breath VOCs can originate from the bloodstream, changes in abundance should also indicate broader physiological effects of exhaustive exercise on the body. Currently, there is limited published data on the effects of exhaustive exercise on breath VOCs. Breath has great potential for biomarker analysis as it can be collected non-invasively, and capture real-time metabolic changes to better understand the effects of exhaustive exercise. In this study, we collected breath samples from a small group of elite runners participating in the 2019 Ultra-Trail du Mont Blanc ultra-marathon. The final analysis included matched paired samples collected before and after the race from 24 subjects. All 48 samples were analyzed using the Breath Biopsy Platform with GC-Orbitrap™ via thermal desorption gas chromatography-mass spectrometry. The Wilcoxon signed-rank test was used to determine whether VOC abundances differed between pre- and post-race breath samples (adjusted P-value $< .05$). We identified a total of 793 VOCs in the breath samples of elite runners. Of these, 63 showed significant differences between pre- and post-race samples after correction for multiple testing (12 decreased, 51 increased). The specific VOCs identified suggest the involvement of fatty acid oxidation, inflammation, and possible altered gut microbiome activity in response to exhaustive exercise. This study demonstrates significant changes in VOC abundance resulting from exhaustive exercise. Further investigation of VOC changes along with other physiological measurements can help improve our understanding of the effect of exhaustive exercise on the body and subsequent differences in VOCs in exhaled breath.

1. Introduction

Despite the numerous benefits of physical activity to human health, participating in exhaustive exercise, such as ultra-marathons, may trigger physiological responses that have a negative impact on the human body. Studies in exercise physiology have suggested an association between running extensive distances with cardiovascular dysfunction, muscle damage, increased susceptibility to upper respiratory tract infections, and severe inflammation [1–4]. Given the increased susceptibility of the respiratory tract to

infections and inflammation, it is reasonable to posit that lung injury may be occurring at or near the alveolar-capillary membrane in elite athletes.

Investigating physiological processes through metabolomic studies in humans has become popular with the advancement of technology in the last decade. Analyzing large datasets of metabolites (generally small molecules <1 KDa) produced by enzymatic activities provides a snapshot of the overall human physiological state. This information allows for the understanding of metabolic changes systemically, as well as providing an opportunity for novel biomarker discovery.

Due to the non-invasive nature of collection and the convenience for repeated sampling within a short period, breath has emerged in recent years as a potential biological matrix for the diagnosis and monitoring of various diseases [5]. The volatile organic compounds (VOCs) contained in human exhaled breath can be derived from a variety of metabolic processes in the body. Given the proximity of the breath to the respiratory tract, VOCs in exhaled breath have been studied for their diagnostic potential in lung diseases such as lung cancer, COPD, pneumonia, and asthma [6–10]. Similarly, VOCs in exhaled breath could be analyzed in response to exhaustive exercise. As most VOCs exchange between the systemic blood and the air in the lungs at the alveolar membrane, exhaled breath is enriched for VOCs generated from physiological processes throughout the body, such as from the liver and the intestines [11–13]. This means that changes in VOC levels in exhaled breath can also provide information on altered metabolic processes deeper in the body than just the immediate respiratory tract. Gases in exhaled breath, such as hydrogen and methane, have demonstrated success in clinical translation as a tool for diagnosing small intestinal bacterial overgrowth and carbohydrate malabsorption [14]. The advantage of studying metabolic changes occurring both proximal and distal to the lungs makes exhaled breath VOCs an ideal tool to better understand the changes occurring under exhaustive exercise.

Currently, very few studies have explored the potential of breath VOCs as biomarkers to reflect metabolic changes induced by exhaustive exercise. These studies measured subject breath VOCs through exhaustive exercise with either a standard ramp-like protocol [15] or a graded maximal exercise protocol [16]. To date, no studies have explored breath VOC changes in the context of running an ultramarathon, where can range from 31–200+ miles.

A limited number of studies have utilized biological samples other than exhaled breath to comprehensively examine the effects of running a marathon on metabolic changes. One study found that within 90 min after the start of a marathon race, free glucose and other stored carbohydrates in human serum can become depleted [17]. Consequently, energy production is most likely switched to the utilization of lipids and amino acids as alternative fuel substrates, suggesting that exhaustive races induce metabolic shifts in the energy-producing pathways [18]. Indications of oxidative stress have also been described [19] when metabolism shifts towards protein catabolism as physical activity surpasses the athlete's lipid stores, or as traditional lipid oxidation pathways become saturated [20]. Alternatively, oxidative stress can also result from cellular inflammatory reactions, which are known to occur in exhaustive exercising [21]. Compared to metabolites in serum that are relatively better understood, specific compounds in breath and their metabolic sources have mostly remained elusive. Regardless, metabolites in serum can provide insights on the metabolic changes underlying exhaustive exercise, which are valuable for hypothesis generation of the potential origins and pathways of VOC's found in breath.

The objective of this study was to evaluate VOCs in exhaled breath that differ in abundance between matched pairs of samples collected before and after the Ultra-Trail du Mont Blanc (UTMB®) ultra-marathon. Breath Biopsy® technology (Owlstone Medical), which allows for VOC biomarker discovery, was utilized in this study to identify potential breath biomarkers of exhaustive exercise. These results will contribute toward a better understanding of the physiological changes that occur during exhaustive exercise.

2. Methods

2.1. Study design and study subjects

The protocol was approved by both the Mayo Clinic Institutional Review Board and the Comite de Protection des Personnes (CPP) Sud-Ouest Et Outre-Mer II. All participants gave written informed consent prior to participation and every aspect of the study conformed to the Declaration of Helsinki and Health Insurance Portability and Accountability Act (HIPAA) guidelines. Thirty-two healthy individuals who participated in the 2019 UTMB ultra-marathon volunteered for the study. The UTMB (171 km, ∼10 000 m ascent) commences in Chamonix, France, between 26 August to 1 September, and courses undulate through alpine regions that remain predominantly above 1000 m with intermittent bouts of altitude exposure over 2500 m. Individual performances of the UTMB are shown supplementary table 1. Participants were asked to visit a dedicated laboratory space for breath collection using the ReCIVA® Breath Sampler (Owlstone Medical) 24−72 h before (pre) and 1–4 h (post) after participating in the UTMB. Due to the difficultly of ultra-trail events, subjects were not requested to fast prior to giving breath samples. However, dietary intake 3 h prior to breath

Table 1. Parameters of TD-GC-MS.

TD-GC-MS method settings	
TD	
Tube desorption temperature (°C)	240
Tube desorption time (min)	5
Trap desorb flow (ml min^{-1})	50
Trap low temperature (°C)	5
Trap heat rate (°C s^{-1})	40
Trap high temperature (°C)	270
Trap desorption time (min)	10
GC	
Column chemistry	TraceGOLD™-624SilMS (6% cyanopropylphenyl-94% dimethylpolysiloxane), 0.25 mm (D) × 1.4 μm (Film thickness) × 30 m (L)
Oven temperature profile	Hold at 37 °C (4 min), 5 °C min^{-1} ramp to 150 °C, 20 °C min^{-1} ramp to 300 °C and hold (10.9 min)
Carrier gas flow (ml min^{-1})	2
Aux heater 1 & 2 temperature (°C)	300
MS	
Scan type	Full
Scan range (m/z)	30–450
Ionization mode	Positive
Electron energy (eV)	35
Transfer line temperature (°C)	300
Ion source temperature (°C)	280

sampling was documented for analysis. A total of 29 volunteers had samples collected pre-race. Two of the subjects did not finish the race and their breath samples were therefore excluded for analysis. In addition, three other subject's sample sets were excluded due to sample curation (93% pass rate). The final analysis consisted of 48 breath samples from 24 subjects, with each subject sampled pre- and post-race.

2.2. Sample collection, analysis and data processing

The Breath Biopsy® platform (Owlstone Medical) was used to standardize the collection of VOCs [22]. Exhaled breath was collected using the ReCIVA Breath Sampler (coupled to a CASPER® Portable Air Supply) by adsorption onto C2-CXXX-5149 bio-monitoring-inert-coated tubes with Tenax TA/carbograph 5TD adsorbent material (Markes International, Llantrisant, UK) through the ReCIVA Breath Sampler. The ReCIVA Breath Sampler monitors subjects' breathing pattern in real time using CO_2 and pressure sensors. Dynamically determined gates using real-time pressure levels trigger the sampling pumps to collect breath. The breath collect method is scaled to a typical tidal breath volume with a start collection threshold corresponding to a CO_2 level of approximately 0.6%. The ReCIVA was configured to collect air from both the upper and lower airways. Before sampling, the tubes were conditioned in a TC-20 (Markes International) by a N2 flow at 20 psi and 320 °C for two hours. The ReCIVA automatically adjusts sample acquisition to each subject's individual tidal volume, and approximately 5.9 l of breath was sampled over 8–12 min in each tube at 225 ml min^{-1}. Ambient contamination was minimized using the CASPER Portable Air Supply. The tubes were purged with helium using a TD-100 (Markes International Ltd Llantrisant, UK). Individual samples were not tested for water removal, instead removal of moisture was assessed by validation of the dry purge performance. Blank sorbent tubes were weighed, then spiked with water. Other tubes were spiked with the OMNI multicomponent QC mix and IS (total 60 compounds). The tubes spiked with water and half of the tubes spiked with QC mix were dry purged, then the tubes that had water added were weighed again. The experiment must demonstrate that the dry purge removed the mass of water but does not remove QC compounds. This test was repeated on dry purge TDs every six months, or after maintenance. Samples were stored at a temperature of 4 °C–8 °C for 8 months before analysis.

Collected samples were analyzed by thermal desorption-gas chromatography mass spectroscopy (TD-GC-MS) using the Breath Biopsy Platform including GC-Orbitrap™. The parameters of TD-GC-MS are presented in table 1. The analysis was followed by feature extraction using Compound Discoverer (v 3.2) (deconvolution of features), see

Supplementary Methods for additional details. A list of features was identified via the in-house Breath Biopsy high resolution accurate mass (HRAM) library of chemicals. The relative abundance of identified VOCs was quantified through comparison to eight deuterated internal standard compounds.

2.3. Statistical analysis

Data were analyzed using the Python programming language (Python Software foundation, Python Language Reference, version 3.10.12 www.python.org/). Data visualization was performed with Matplotlib (3.7.1), seaborn (0.12.2) and plotly (5.16.1).

The relationship between demographic variables (age, BMI, sex, percent weight, and diet) and identified VOCs was examined using principal component analysis (PCA), a method for reducing data dimensionality to visually investigate the underlying data structure.

To determine whether the peak area of each VOC differed significantly between pre- and post-race breath samples, we used the Wilcoxon signed-rank test, a non-parametric paired test. VOCs with an adjusted *P*-value of less than .05 were considered to have strong evidence of an association with exhaustive exercise. The Benjamini-Hochberg False Discovery Rate was used to adjust *P*-values for multiple testing.

VOCs of interest were also assessed in blank samples of the ambient air surrounding the participant at the point of breath collection (ambient blank; n = 3) and equipment blanks; n = 5). This analysis was performed qualitatively rather than by statistical analysis due to the small number of each type of blank sample. It is expected that breath VOCs that are truly originated from the breath and are not an environmental contaminant compound will have an average concentration in subjects that is at least three standard deviations above the value(s) found in any type of blank sample.

3. Results

3.1. Demographic distribution

The demographics of this study are presented in table 2. Out of the 24 participants who took part in the ultra-marathon, 21 (87.5%) were male. The median age of all participants was 38.8 (±8.9). The participants' median BMI was 22.2 (±2.1) pre-race and 21.5 (±2.1) post-race. The drop of BMI, which was contributed by weight loss, was likely due to dehydration. None of the subjects had detectable halitosis pre-race, whereas five subjects (20.8%) had detectable halitosis post-race. The information of halitosis was recorded through either the breath collection operator asking the subjects or a noticing of breath odor at breath sampling. The five subjects with detectable halitosis post-race did not show different clustering compared to the remaining subjects in PCA analysis (supplement figure 1). Food and drinks consumed within three hours before breath sampling were recorded. Consumption of coffee, citrus drinks and fruits were paid with special attention, as these foods and drinks have specific VOCs that can be detected in breath (PCA analysis in supplement figure 1). In this study, a total of 15 (62.5%) and 8 (33%) subjects consumed coffee/citrus drinks and fruit pre-race, respectively. 12 (50%) and 11 (45.8%) subjects consumed coffee/citrus drinks and fruit post-race, respectively.

Table 2. Demographics of the 24 subjects that participated in the 2019 UTMB.

Total Participants	N = 24	
Gender (Male)	N = 21 (87.5%)	
Median Age	38.8 (±8.9)	
	Pre-race	Post-race
Median BMI	22.2 (±2.1)	21.5 (±2.1)
Halitosis Detection	N = 0	N = 5 (20.8%)
Coffee/Citrus Drinks	N = 15 (62.5%)	N = 12 (50%)
Fruit Consumption	N = 8 (33%)	N = 11 (45.8%)

3.2. PCA

To determine if there were differences in VOC profiles between breath samples collected before and after the race, PCA was conducted to examine the high-level data structure (figure 1, supplement figure 2). The results indicated that breath VOCs between pre- and post-race collections were slightly separated by principal component 1 (PC1) and PC2. Given the presence of 793 different VOCs in a breath sample, it is not uncommon for large datasets similar to the one in this study to exhibit modest separation between different groups. The separation of breath VOCs between pre- and post-race samples was more evident in PC3 and PC4, suggesting that while race-relevant effects were not the most significant source of variation across all samples, they were a crucial factor in the differences of VOC abundance observed before and after the race. Despite the modest separation in PC1 and PC2, important VOCs that may reflect physiological changes in exhaustive exercise were identified in the dataset with further analysis between pre- and post-race.

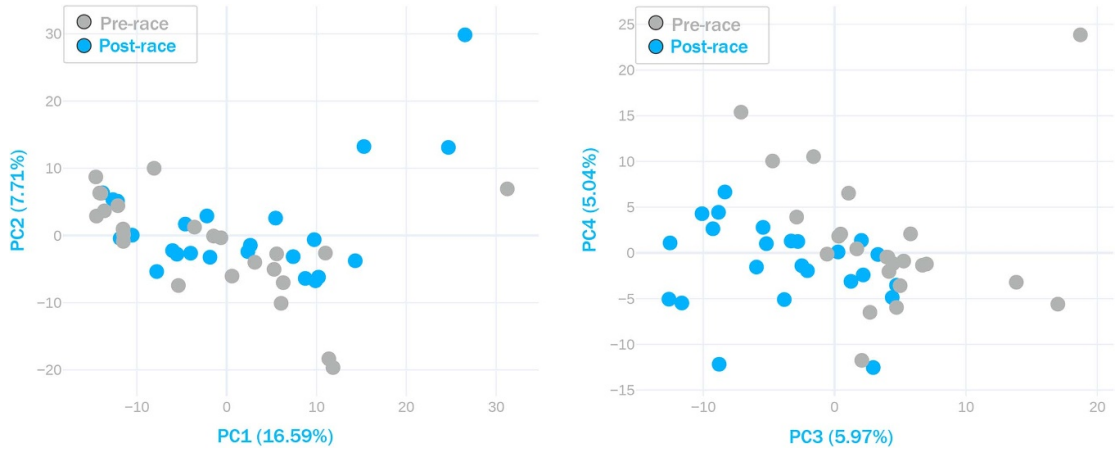

Figure 1. PCA plots showing separation between pre- and post- race samples (left, PC1 vs PC2; right, PC3 vs PC4).

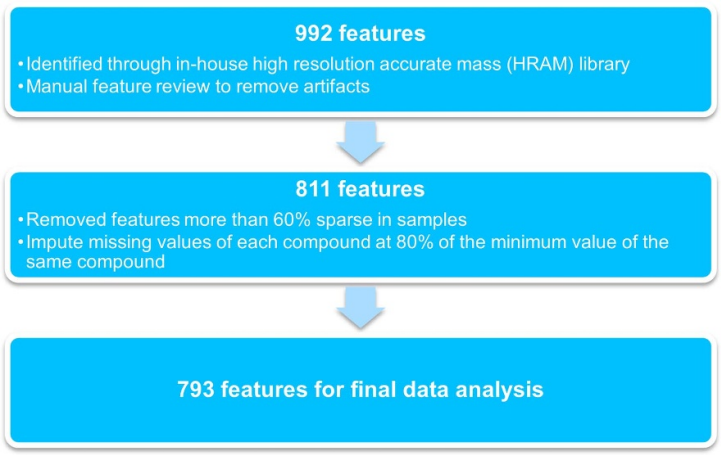

Figure 2. A flow chart of the data analysis process.

3.3. Overview of the VOC dataset

Figure 2 presents a flow chart of the data analysis process. A total of 992 features were present in the dataset. After feature extraction and quality control, a total of 811 different features were identified. To reduce noise in the data, features that were present in only a small fraction of the samples were removed, and those that were present in 40% or more of the total samples were kept. Missing values within each feature were imputed at 80% of the minimum value of that same feature. This resulted in 793 features for further analysis. Among these features, 74 features were assigned with a VOC compound identity based on comparisons to the Breath Biopsy HRAM Reference Library. After correcting for multiple testing (Benjamini—Hochberg False Discovery Rate, *P*-value <.05), 63 VOCs showed significant differences between pre- and post-race samples (12 decreased and 51 increased) (figure 3). A complete list of the significantly different VOCs is shown in supplementary table 2.

3.4. VOC IDs with a significant difference in post-race samples

While the true identification of a given VOC requires a matching reference standard alongside samples, VOCs identified through untargeted analysis could be interpreted with information such as chemical class and a tentative ID assigned by available compound libraries. Among the 63 VOCs that reached statistical significance in the pre- and post-race comparisons, 15 VOCs were putatively annotated from the Breath Biopsy HRAM Library according to the Metabolomics Standards Initiative (MSI) guidelines [23] (table 3). These compounds belong to the chemical classes of ketones, alcohols, alkenes, carboxylic acids, carboxylic esters and diazoles.

3.5. Comparison of breath VOCs with blank samples

The identification of VOCs that reached statistical significance between pre- and post-race samples may

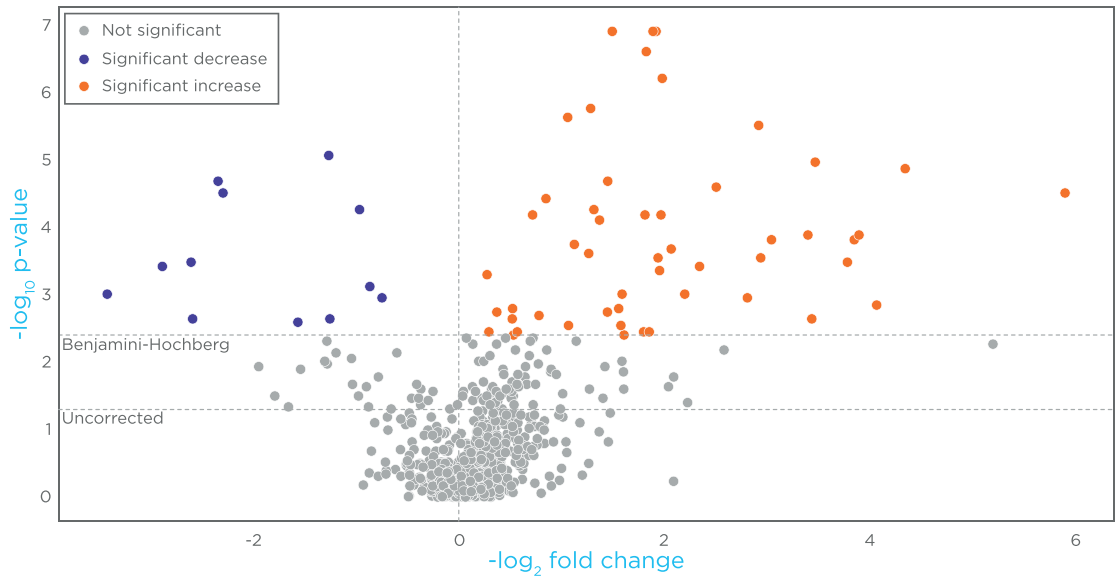

Figure 3. Volcano plot of high-level VOC changes between pre- and post-race samples. A total of 63 VOCs reached statistical significance (Benjamini—Hochberg adjusted *P*-value $< .05$) between pre- and post-race samples (12 decreased colored in purple and 51 increased colored in orange).

Table 3. A list of significantly changed VOCs between pre- and post-race samples with a putatively annotated (MSI level 2) compound name.

Compound name	Chemical class	RI	Pre-race median	Post-race median	Median Fold change	Multiple testing corrected *P*- value
Acetate	Carboxylic Acid	695	0.008	4.0	59.3	0.001
Acetone	Ketone	525	74.12	636.13	7.5	0.000 03
Isoprene	Alkene	512	3.51	14.40	4.7	0.02
2,3-Butanedione	Ketone	628	0.04	0.11	3.9	0.0002
Isopropyl Alcohol	Alcohol	534	2.77	8.95	3.8	0.0007
2,3-Butanediol	Alcohol	879	0.004	0.009	2.8	0.000 03
4-Heptanone	Ketone	908	0.003	0.008	2.7	0.03
Methyl Vinyl Ketone	Ketone	625	0.27	0.76	2.4	0.0006
2-Butanone	Ketone	633	1.03	1.83	1.8	0.0002
2-Pentanone	Ketone	732	0.010	0.017	1.6	0.0002
Methyl Formate	Carboxylic Ester	—	3.92	8.34	2.08	0.0003
Methyl Acetate	Carboxylic Ester	546	8.4	33.44	3.94	0.0001
2,3-Butanediol (isomer)	Alcohol	871	0.003	0.009	3.70	0.000 03
1,4-Dimethylimidazole	Diazole	1067	0.000 01	0.001	16.67	0.02
1,5-Dimethylimidazole	Diazole	1167	0.0001	0.001	7.64	0.01

indicate important metabolites that are related to the underlying physiological changes in exhaustive exercise. However, distinguishing breath VOCs derived from metabolic processes in the body versus those present in the ambient air that have been inhaled is crucial to truly capture the physiological changes. This study incorporated both ambient air and equipment blank samples. Although the number of blank samples were not sufficient for statistical analysis, the levels of VOCs captured in the blank samples provide a baseline to facilitate the interpretation of an 'on-breath' VOC, which can be reliably distinguished from background signal While three VOCs, isopropyl alcohol, hexamethyldisiloxane, and 2-pentanone were significantly increased in post-race samples (table 3), the median levels were not above the levels observed in blank samples (supplement figure 3). Therefore, these VOCs may not represent physiological changes in the human body.

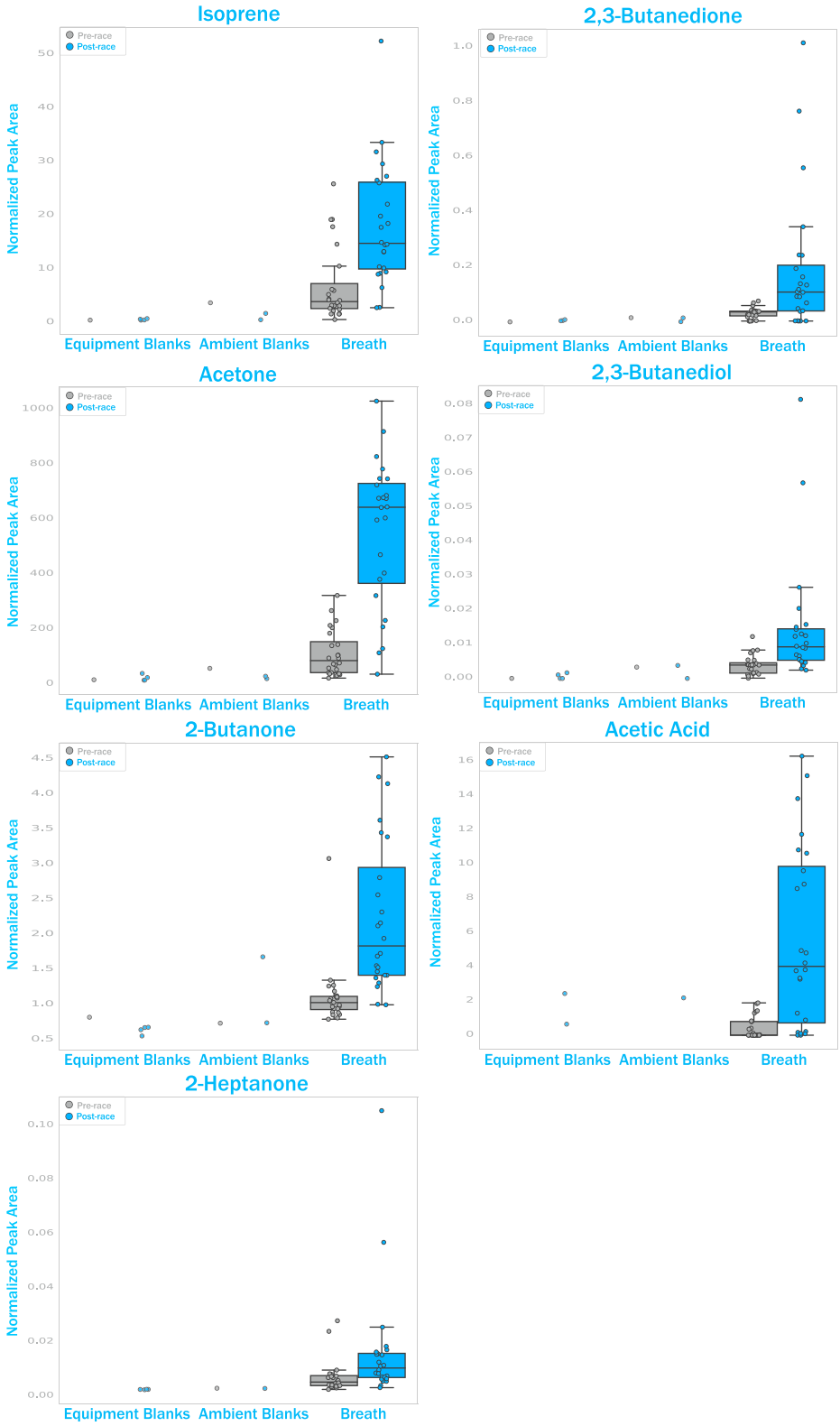

Figure 4. VOCs significantly changed post-race that are associated with physiological changes in exhaustive exercise.

3.6. VOCs reflecting physiological changes in exhaustive exercise

Upon considering the median levels of VOCs above blanks and the current understanding of their role in biology, seven VOCs that significantly increased in post-running samples are suggested to reflect the different underlying physiological changes in exhaustive exercise. These VOCs are acetate (fold change = 59.3, *P*-value = .001), acetone (fold change = 7.53, *P*-value = .003), isoprene (fold change = 4.58, *P*-value = .02), 2,3-butanedione (fold change = 3.9, *P*-value = 0.002), 2,3-butanediol

(fold change = 2.81, P-value = .00003), 4-heptanone (fold change = 2.72, P-value = .003) and 2-butanone (fold change = 1.8, P-value = .002) (figure 4). Together, these seven VOCs suggest the involvement of fatty acid oxidation, inflammation, and possible altered gut microbiome activity in response to exhaustive exercise.

4. Discussion

This study provides several important advances in the field. First, it shows that exhaled breath VOCs in post-race samples differ from pre-race samples in ultramarathon runners. While studies have suggested metabolic changes induced by exhaustive exercise using human serum, few have been undertaken using exhaled breath VOCs. The studies that have measured breath VOCs all used an exercise protocol that exhausts subjects acutely [15, 16], and focused heavily on changes in acetone and isoprene, which are two of the most abundant VOCs in human breath. This is the first study to explore metabolic changes using exhaled breath VOCs in ultramarathon runners, with a broader discussion on the physiological processes that breath VOCs may reflect. The processes implicated include fatty acid oxidation and inflammation, which aligns with other metabolic studies using human serum, as well as possible altered gut microbiome activity. The findings also suggest that breath biomarkers, along with their non-invasive nature, have great potential and are likely to be a preferred method to help athletes monitor their health compared to blood sampling.

Similar to other studies that have explored breath VOCs in exhaustive exercise, isoprene and acetone, two of the most highly abundant and perhaps the most extensively studied VOCs in exhaled breath, were found to be significantly different in post-race runners in this study. Isoprene is a metabolic by-product of cholesterol biosynthesis through the mevalonate pathway [24]. Through the identification of its extrahepatic source, stored isoprene has been proposed to be released from working muscles at the onset of exercise [25]. Acetone, the simplest ketone, is a product of acetoacetate decarboxylation (derived through lipolysis) [26]. Unlike the increased levels of isoprene and acetone found in post-race runners in this study, literature studies show mixed results regarding exhaled breath isoprene and acetone levels after exhaustive exercise. However, comparisons across different studies should be made with caution, as several factors could contribute to the mixed findings, including differences in study design (i.e. exercise intensity and duration), dietary restrictions, VOC background levels, subject demographics and cardiorespiratory fitness [15, 16, 27–30]. These are also some of the factors that challenge the data reproducibility in breath research, despite a gradual interest in the last few decades to utilize breath VOCs as potential biomarkers. While some of these factors may be challenging to control, others could be addressed through the standardization of breath collection process, such as collected volume, collected breath fractions (i.e. from the upper respiratory tract versus from deeper in the lungs), and the utilization of filtered air and blank samples to distinguish breath VOCs from background noise and eliminate spatial and temporal variabilities from the environment. With these steps considered, the reproducibility of findings in exhaled VOCs can be improved. In addition to the increased levels of acetone, two other ketones, 2-butanone and 4-heptanone, were found to be increased in post-race runners compared to pre-race. The production of ketones has been demonstrated from (dietary) lipid oxidation under heat, as well as conversion from aldehydes [31]. The production of these methyl ketones could be a result of utilizing lipids as an alternative energy source during exhaustive exercise due to increased energy demand. It is also possible that increased levels of these methyl ketones suggest the presence of inflammation, as inflammation is associated with lipid oxidation. Correlation analysis of these methyl ketones with additional physiological parameters will suggest the associations of specific physiological processes undergone in exhaustive exercise. Given the proximity of breath to the respiratory tract, it is likely that these VOCs reflect inflammation occurring at or near the alveolar-capillary membrane due to lung injury. However, we do not exclude the possibility of inflammation occurring elsewhere in the body, as exhaled breath also carries VOCs from the bloodstream.

In this study, post-race runners exhibited the greatest increase acetate levels among all other VOCs. Acetate, the most abundant short chain fatty acid (SCFA) in the human body, plays an important role as a metabolic substrate. Gut microbial fermentation can produce acetate, which, when not fully absorbed by the intestine, enters the portal vein to be taken up by the liver or released into the circulatory system. In the liver, acetate can be converted to acetyl-CoA, where can be used as a substrate for fatty acid synthesis or for use in the TCA cycle. Under extreme fasting conditions or during exhaustive exercise when carbohydrate energy fuels are depleted, fatty acids can be oxidized in the liver to generate acetate [32]. Increased levels of breath acetate have been found after prolonged exercise [27]. In animal models of endurance exercise, mice administered with antibiotics, or a low microbiome-accessible carbohydrate diet demonstrated significantly reduced endurance capacity that can be restored by infusion of acetate or fecal microbiota transplantation [32]. This demonstrates that intestinally-produced acetate is an important energy source during prolonged exercise,

and is arguably the most important SCFA energy source for skeletal muscle in general.

Consistent with acetate, the increased levels of 2,3-butanedione and 2,3-butanediol, both by-products of microbial fermentation, support the effects of exhaustive exercise on the gut microbiome. The compound 2,3-Butanedione is formed via the thiamine pyrophosphate-mediated condensation of pyruvate and acetyl CoA in some fermentative bacteria [33, 34], whereas 2,3-Butanediol is produced by pyruvate and NADH.

The involvement of VOCs methyl vinyl ketone, methyl formate, methyl acetate, 1,4-dimethylimidazole, 1,5-dimethylimidazole and their potential biological roles in exhaustive exercise remain unknown (table 3, supplement figure 4). These VOCs all exhibit changes that reached statistical significance in post-race samples with very low levels (1,4-dimethylimidazole and 1,5-dimethylimidazole were even below the detection limit) in blanks. More research on the biochemistry of these VOCs is needed to help better understand their roles in human physiology.

In this study, 2-pentanone, a biomarker of inflammation found associated with several diseases [35–37], and isopropyl alcohol (also known as 2-propanol), a potential biomarker of lipid peroxidation [38], were found significantly increased in post-race samples (table 3, supplement figure 3). However, the median levels of 2-pentanone and isopropyl alcohol in pre- and post- race breath samples were less than the observed levels in pre- and post-race blank samples. This suggests that the levels of these two VOCs found in breath samples were not solely produced from endogenous metabolism but were being contributed, at least partially, by background. Hexamethyldisiloxane exhibited a similar result to 2-pentanone and isopropyl alcohol (table 3, supplement figure 3). However, hexamethyldisiloxane is a silicon-containing compound that is unlikely to be an endogenously produced metabolite. Hexamethyldisiloxane is suggested to originate from various sources, including personal care products such as antiperspirants, cosmetics and hair-care products [39], and may also derive from the sorbent materials used to capture VOCs or from silicone-containing tubing and seals present in VOC capturing equipment [40, 41]. These findings suggest that the analysis of breath samples and the identification of a VOC that truly reflects physiological changes must be undertaken with caution. Background levels of a VOC of interest, its potential origin sources, and the understanding of its relation to biology must all be taken into careful consideration.

Generally, it is necessary to control dietary intake and drinks in moderation prior to breath sampling due to the complexity of distinguishing what compounds may or may not influence metabolic changes. However, it is unrealistic and unreasonable to request subjects to fast in this study, therefore, dietary intake and drinks taken were recorded but not restricted (table 2). There is evidence of some clustering in the PCA plot for coffee consumption in the pre-race samples, suggesting drinking coffee has some impact on breath VOCs (supplement figure 1). However, too few of the subjects drank coffee last in the post-race samples, therefore it is most likely that compounds relevant to coffee would have been excluded given the number of subjects who had coffee between pre- and post-race. In the final dataset, we did not identify any VOCs that reached statistical significance and with an assigned ID likely from drinking coffee (such as compounds derived from the Millard reaction due to roasted beans). We found no significant clustering from the subject's last drink containing citrus or fruit consumption. In the final dataset, we did not identify any VOCs that reached statistical significance and with an assigned ID likely from citrus fruit or drinks (such as terpenes like limonene).

There are several limitations to this study. The majority of the participants were male, which may limit generalizability of the results. The varied times of post-race breath sampling due to the differences of completion pace and post-race activities were quite difficult to control. While post-race breath sampling times were distributed quite evenly between one to three hours after the end of race, this likely contributed to the subtle differences of VOC profiles observed between pre- and post-race samples. Several other things in this study that could be important yet still difficult to control due to the nature of this study include age, elite versus non elite runners, body composition, race times, recovery times, hydration levels, training levels and cultural backgrounds. While ambient and instrument blanks were included in the study, the number of blank samples were not sufficient for statistical analysis to be conducted. Additionally, because the blank samples were not paired per subject, they may not reflect the background levels across the entire period of sample collection. The sample size of this study was also small, therefore, a validation study with a larger, more balanced cohort with paired blanks should be conducted to ensure the robustness of the results.

5. Conclusion

This study demonstrates that there are differences in exhaled breath VOCs between pre-race and post-race ultramarathon runners, potentially reflecting various physiological responses to exhaustive exercise, such as alternative energy fuel sources, inflammation, and changes in the gut microbiome activity. Moving forward, the findings from this pilot study should be validated in a larger and more diverse cohort with a similar study design. Moreover, additional research on

VOC changes, along with other physiological measurements, could provide further insight into the biological associations between VOCs and exhaustive exercise.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Ethical statement

The protocol was approved by both the Mayo Clinic Institutional Review Board and the Comite de Protection des Personnes (CPP) Sud-Ouest Et Outre-Mer II. All participants gave written informed consent prior to participation and every aspect of the study conformed to the Declaration of Helsinki and Health Insurance Portability and Accountability Act (HIPAA) guidelines.

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