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Hyperbaric oxygen diving affects exhaled molecular profiles in men

P.J.A.M. van Ooij[a,b,*], R.A. van Hulst[a,c], W. Kulik[d], P. Brinkman[b], A. Houtkooper[a], P.J. Sterk[b]

[a] Diving Medical Center, Royal Netherlands Navy, Den Helder, The Netherlands
[b] Department of Respiratory Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[c] Laboratory of Experimental Intensive Care and Anesthesiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[d] Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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ABSTRACT

Exhaled breath contains volatile organic compounds (VOCs) that are associated with respiratory pathophysiology. We hypothesized that hyperbaric oxygen exposure (hyperoxia) generates a distinguishable VOC pattern. This study aimed to test this hypothesis in oxygen-breathing divers. VOCs in exhaled breath were measured in 10 male divers before and 4 h after diving to 9 msw (190 kPa) for 1 h. During the dive they breathed 100% oxygen or air in randomized order. VOCs were determined using two-dimensional gas chromatography with time-of-flight mass spectrometry. Compared to air dives, after oxygen dives there was a significant increase in five VOCs (predominately methyl alkanes). Furthermore, a strong, positive correlation was found between increments in 2,4-dimethyl-hexane and those of 4-ethyl-5-methyl-nonane. Although non-submerged hyperoxia studies on VOCs have been performed, the present study is the first to demonstrate changes in exhaled molecular profiles after submerged oxygen diving. The pathophysiological background might be attributed to either a lipid peroxidation-induced pathway, an inflammatory pathway, or to both.

1. Introduction

Since the first analyses of exhaled air in the 1960s, more than 3000 volatile organic compounds (VOCs) have been detected in exhaled breath, which can arise from a pulmonary and/or systemic origin (Anderson and Hlastala, 2007; Paredi et al., 2002; Phillips et al., 1999). Of these compound, about 1% are likely to contain disease-specific VOCs (Pennazza et al., 2010), most of which are alkanes, isoprenes, benzenes and methyl alkanes (Mazzone, 2012; Phillips et al., 1999). Using techniques like gas chromatography–mass spectrometry (GC–MS) or absorption spectrometry, these VOCs can be measured in exhaled breath samples and a VOC profile can be produced, also called a VOC breath print (Röck et al., 2008). Several breath prints have been identified which can be used for the assessment of diseases like lung cancer (Dragonieri et al., 2009; Peng et al., 2009; Phillips et al., 2003), infectious diseases like tuberculosis (Phillips et al., 2012), and inflammatory conditions such as asthma (Dragonieri et al., 2007; Fens et al., 2009) and chronic obstructive pulmonary disease (COPD) (Fens et al., 2009, 2011).

Breathing oxygen with a partial oxygen pressure (P_{O_2}) between 50 kPa and 300 kPa (hyperoxia) introduces pathological changes known as pulmonary oxygen toxicity (POT), which manifests itself as atelectasis, (interstitial) edema, inflammation and, finally, as fibrosis (Clark and Lambertsen, 1971). Due to the formation of radical oxygen species (ROS) pathological phenomena like lipid peroxidation of the alveolar cellular membrane and activation of inflammatory cells can be found in the early development of POT in both humans and animals (Bostek, 1989; Fisher et al., 1984; Morita et al., 1986). As both processes generate VOCs, divers who breathe higher inspired oxygen fractions are likely to develop a specific hyperoxic-induced VOC breath print.

Only a few studies have measured VOCs before and after oxygen exposure. Moreover, most of these studies were performed in healthy subjects under normobaric, non-immersed circumstances with a varying range of P_{O_2} (28 kPa to 100 kPa) and exposure time (30–125 min). In only one study divers underwent a non-immersed hyperbaric exposure breathing oxygen with P_{O_2} of 42 kPa to 50 kPa (Suzuki, 1994). The most frequently observed changes in VOCs after oxygen exposure were an increase in alkanes (e.g. pentane, ethane and methyl alkanes) (Loiseaux-Meunier et al., 2001; Morita et al.,

* Corresponding author at: Diving Medical Center, Royal Netherlands Navy, PO Box 10.000, 1780 CA Den Helder, The Netherlands. Tel.: +31 223 653076; fax: +31 223 653148.
E-mail addresses: pjam.v.ooij.01@mindef.nl, p.j.vanooij@amc.uva.nl (P.J.A.M. van Ooij).

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1986; Phillips et al., 2003; Suzuki, 1994) although changes in pentane were not observed after breathing 60% oxygen (P_{O_2} = 60 kPa) for 30 min (Lemaitre et al., 2002).

These findings challenge the concept that breathing 100% oxygen at 101 kPa for less than 6 h is too short a period to generate a POT effect based on either clinical signs or symptoms in healthy volunteers (Tinits, 1983). This implies that POT may even develop at relatively low P_{O_2} levels. As increments in specifically alkanes and methyl alkanes have been found, lipid peroxidation could be a prodromal stage for POT (Morita et al., 1986). Therefore, measuring a specific VOC breath print could be a sensitive method to monitor the early onset of POT. As the onset of POT develops earlier in submerged oxygen diving (van Ooij et al., 2011) it can be hypothesized that even short oxygen dives generate a specific VOC breath profile. However, to our best knowledge this hypothesis has not yet been tested in submerged hyperbaric oxygen exposures such as routine oxygen dives.

Based on the above we hypothesized that submerged hyperbaric oxygen-exposed divers will develop a distinctive VOC breath print. As lipid peroxidation is thought to be an early POT mechanism we focused on changes in alkanes and methyl alkanes.

2. Methods

2.1. Subjects

All participants included in this study were certified professional divers of the Royal Netherlands Navy (Den Helder, NL). They were all medically certified as 'fit to dive' based on absence of symptoms, normal spirometry and exercise testing (Wendling et al., 2004). Exclusion criteria were history of recent lower respiratory tract infection and usage of any medication. As a result, 10 male divers (2 smokers, 1.9 and 4.8 pack years, respectively) voluntarily participated in the present study.

2.2. Ethical considerations

This study was approved by the Academic Medical Ethics Review Board (Academic Medical Center Amsterdam, NL). All participants gave written consent after being informed about the aims of the study and having received written information. They could withdraw from the study at any time and the study results would not be included in their personal medical file. The study was registered at www.trialregister.nl under NTR3106.

2.3. Dive protocol and procedures

The chosen dive protocol was a dive to 9 meter of seawater (msw) with a dive time of 60 min. The oxygen load derived was based on the unit of pulmonary toxicity dosage (UPTD) model by Bardin and Lambertsen (1970). In this model, one UPTD was the degree of pulmonary poisoning produced by breathing 100% oxygen continuously at 101 kPa (1 atmosphere absolute) for 1 min. For the oxygen dives the UPTD load was estimated at 142 UPTDs, which fell within the maximal daily limits of 615 UPTDs as advised by the US Navy (Arieli et al., 2002). As predicted by the UPTD model this oxygen load would generate POT effects that are equivalent of breathing 100% oxygen at atmospheric pressure for 142 min.

Diving itself was performed in the wet compartment of our dive simulator (Medusa, Haux Life Support, Germany) at Den Helder. No physical activity was performed during any dive and all dives were performed in the prone position. For all dives the water temperature was 15 °C–20 °C.

2.4. Design

This study had a randomized, double-blind crossover design. Each subject was monitored during two separate days on which a dive was made whilst breathing either compressed air or 100% oxygen in randomized order. In the present study these days are referred to as 'air diving day' when compressed air was used as breathing gas or 'oxygen diving day' when 100% oxygen was breathed.

On each study day the subject was measured twice. Measurement clock times were the same on both days: baseline exhaled breath samples were taken between 08^{00} and 09^{00} while the dive itself took place between 10^{00} and 12^{00}. Exhaled breath was again sampled 4 h after the dive, based on the expected maximal POT effects described in previous studies (van Ooij et al., 2009, 2011, 2012). To determine the amount of oxidative stress that the subjects encountered, blood levels of malondialdehyde (MDA) were measured (Loiseaux-Meunier et al., 2001). Baseline venous samples were taken between 08^{00} and 09^{00} while the post dive samples were taken 30 min after the dive.

On these study days all measurement values acquired between 08^{00} and 09^{00} are referred to as 'Pre' values, whereas the subsequent post dive measurements are referred to as 'Post' values.

Between each measurement day, a transitional period of at least 7 days was taken into account. Participants were not allowed to drink coffee, eat heavy meals, do heavy physical exercise (i.e., sports activities, assault courses, etc.) or smoke within 1 h prior to any measurement. In addition, they were not allowed to dive within 3 days prior to any measurement day.

2.5. Equipment and measurements

All divers wore a dry suit (Viking, Trelleborg Protective Products AB, Sweden) with a woollen undergarment and a full-face mask (Divator, Interspiro, Sweden). The breathing equipment itself was an adapted surface supplied open circuit equipment (Divator, Interspiro, Sweden), which was connected to a switchboard on the dry part of the wet compartment. Based on randomization the diver was supplied through this switchboard with either compressed air or 100% oxygen. The diver himself was unaware of which type of gas he was breathing; however we cannot rule out the possibility that oxygen 'tasted' differently, thereby perhaps enabling the diver to discriminate between the breathing gases. The Divator work of breathing is less than 3 J s^{-1} at 50 msw in accordance with the EN250 guidelines while the positive work of breathing during inhalation is $\leq$0.3 J l^{-1} (ECS, 2000). These maximal values were not reached in the dive depth used in the present study.

2.6. Exhaled breath

2.6.1. Sampling

Exhaled air sampling was done by a validated method as previously described (Dragonieri et al., 2007; Fens et al., 2009). Briefly, with the nose clipped, the subjects breathed through a mouthpiece connected to a 2-way non-rebreathing valve with an inspiratory VOC filter (A2, North Safety, Middelburg, the Netherlands) and an expiratory silica reservoir. Using an inspiratory VOC filter prevents environmental VOCs from contaminating exhaled breath samples. After 5 min of equilibration by tidal breathing of VOC-filtered air the subject performed one inspiratory capacity manoeuvre and exhaled a vital capacity volume into a 10 l Tedlar bag (Dragonieri et al., 2007; Fens et al., 2009). Within 10 min after collection of the exhaled breath sample, the content of the Tedlar bag was transported by a vacuum pump (0.5 l at a flow rate of 200 ml s^{-1}) into a stainless steel absorption tube conditioned with Tenax GR (Gerstel, Müllheim a/d Rurh, Germany). Before and after loading with

breath samples the tubes were capped airtight. The loaded absorption tubes were stored at 8 °C until analysis.

2.6.2. Analysis

Exhaled breath samples were analyzed with a chromatography with time-of-flight mass spectrometry (GC–TOFMS) (Pegasus GC–TOFMS, LECO Corp., St Joseph, MI, USA) equipped with a Rxi-1 column of 30 m × 0.25 mm × 0.25 μm (Restek, Bellefonte, PA, USA). Pure GC-grade Helium (He) was used as carrier gas at a constant flow rate of 1 ml min^{-1}. Tenax tubes containing exhaled breath samples were desorbed in a Gerstel Thermal Desorption System (TDS). When a tube was loaded in the TDS, the temperature was 0 °C. After the tube was purged with He during 1 min, the tube was heated at a rate of 60 °C min^{-1} to 250 °C where it was hold for 10 min for thermal desorption of the tube. During desorption the TDS was in splitless state. Compounds desorbed from the tube were trapped in the Gerstel Cool Injection System (CIS4) cold-trap containing a liner packed with glass wool at −100 °C. During desorption of the tube the CIS4 was in a Solvent Vent state with a flow of 50 ml min^{-1}. After 10 min of desorption the tube was replaced by an empty one, after which the temperature was rapidly increased to 250 °C (hold 5 min) with 720 °C min^{-1} for the analyte to transfer to the column. Transfer mode was splitless during 1.5 min. The GC oven ramp temperature was programmed from 40 °C to 310 °C with a rate of 10 °C min^{-1}. At 310 °C it was hold for 5 min. The MS transfer line temperature was 250 °C and the ion source temperature was 200 °C with EI energy of 70 eV. The collected mass range was 29–450 amu with an acquisition rate of 20 spectra s^{-1}. The data were processed with LECO ChromaTOF-GC version 4.50 for automatic peak find and true signal deconvolution at a defined signal-to-noise threshold of 100:1. Data alignment was done using LECO's Statistical Compare feature of ChromaTOF.

2.7. Malondialdehyde (MDA)

2.7.1. Blood sampling

Venous blood was sampled from the cubital vein into 10 ml-sample tubes containing EDTA. The space above the blood sample was replaced by nitrogen. After centrifugation (1100 g for 10 min) the samples were immediately stored at −30 °C until analysis. For the measurement of MDA venous blood plasma was used.

2.7.2. Analysis

MDA ($CH_2(CHO)_2$) is a highly reactive dialdehyde and is used as a marker for oxidative stress. In the present study, MDA was measured using an improved method as described by Pilz et al. (2000). Briefly, total (free and bound) MDA was determined as the 2,4-dinitrophenylhydrazine (DNPH) derivative. After addition of the stable isotopically labeled analogue ($^2H_2$-MDA) as internal standard (IS), alkaline hydrolyzation, deproteinization and derivatization with DNPH, MDA-hydrazone was analyzed by ultra-performance liquid chromatography mass spectrometry (UPLC–MS/MS) and positive electrospray ionization. Using an Acquity UPLC system (Waters Corperation, Milford, USA) samples were injected on a LC-18-DB analytical column (250 × 4.6 mm, 5 μm particles, Supelco) hyphenated to a Quattro Premier XE mass spectrometer (Waters Corporation, Milford, USA). Analyses and IS were eluted with acetonitrile/water/acetic acid (50/50/0.2) and detected in multiple reaction monitoring (MRM) mode for the transitions *m/z* 235 → *m/z* 159; *m/z* 237 → *m/z* 161. Samples were quantified against calibration standards.

2.8. Statistical analyses

The data used were the Area Under the Curve (AUC) of each of the VOCs. As VOC data have a tendency toward non-normal distribution (Formenton et al., 2013; Jia et al., 2008) the results were log transformed. Despite this log transformation, some of the data were still non-normally distributed, although to a lesser degree. To determine the changes between pre and post diving for all parameters (delta (Δ) values) we subtracted the (log transformed) *Pre* value from the (log transformed) *Post* values. The Wilcoxon sign rank test was used to compare the Δ values of oxygen dives and air dives. Kendall's rank correlation coefficients (Kendall's τ_α) (Newson, 2002) were calculated to examine the relationship between each of the Δ VOCs and the relationship between Δ VOCs and Δ MDA. A p-value <0.05 was considered significant. The results are presented as median and interquartile range (IQR). Analyses were performed using R software (R Foundation for Statistical Computing, version R i386 2.15.0, Austria) and Stata SE software (version 9.2, StataCorp, USA).

Table 1
Demographic data of the included divers (n = 10).

	Mean	SD
Age (years)	31.4	6.6
Height (cm)	184	6
Weight (kg)	89.2	7.9
Lean body mass (%)	84.4	3.7

3. Results

A total of 10 male divers were included (Table 1). Due to a technical problem, blood sampling failed in one diver; therefore, blood was sampled during 10 oxygen dives and 9 air dives. There were no problems regarding the sampling of exhaled breath. None of the divers reported any clinical symptoms or signs of pulmonary oxygen toxicity after the oxygen dives before blood- or exhaled breath sampling.

Based on a signal-to-noise threshold of 100:1 a total of 321 VOCs were identified in both the oxygen diving group and air diving group. From these 321 VOCs significant differences in Δ value between oxygen dives and air dives were found in five VOCs. Among these five VOCs there were no significant differences in the baseline values, except for 2-methyloctacosane. At baseline, this latter VOC was only observed in the air diving group and not in the oxygen diving group (Table 2). Three of these five VOCs were identified as methyl alkanes. In the oxygen diving group four out of five VOCs had a positive Δ value implying increased post-dive values as compared to pre-dive. In contrast, in the air diving group all of these VOCs had a negative Δ value. No significant changes in other alkanes (like ethane and pentane) were found in either the oxygen diving group or the air diving group (Table 3). Kendall's τ_α rank correlation coefficient between 2,4-dimethyl-hexane and 4-ethyl-5-methyl-nonane was 0.73 with a p value <0.001. The 95% confidence interval implies that it is 45%–100% more likely that a higher value of 2,4-dimethyl-hexane comes with a higher value of 4-ethyl-5-methyl-nonane

Table 2
Median and interquartile range (IQR) pre-dive value of volatile organic compounds (VOC) of oxygen and air diving group. Values are area under the curve (AUC).

VOC	Oxygen pre dive (IQR)	Air pre dive (IQR)	p=
2-Methyloctacosane	No observations	14.05 (1.29)	*
Methyl-cyclohexane	15.21 (0.47)	15.26 (0.61)	0.78
4-Ethyl-5-methyl-nonane	13.51 (0.11)	13.25 (0.57)	1.00
2,4-Dimethyl-hexane	13.67 (1.35)	14.88 (0.24)	0.08
1,3,5,7-Cyclooctatetraene	14.84 (0.70)	15.08 (0.90)	0.20

p-value = significance level oxygen dive compared with air dive. VOCs printed **bold** are methyl alkanes.
* Stata could not calculate p-value.

Table 3
Median and interquartile range (IQR) Δ value of volatile organic compounds (VOC) after air and oxygen dives. Values are area under the curve (AUC).

VOC	Δ Oxygen dive (IQR)	Δ Air dive (IQR)	p=
2-Methyloctacosane (MOC)	14.60 (1.24)	−13.45 (13.29)	0.017
Cyclohexane, methyl- (MCH)	0.02 (1.46)	−1.02 (2.22)	0.047
Nonane, 4-ethyl-5-methyl- (EMN)	0.00 (0.31)	−12.11 (12.97)	0.005
Hexane, 2,4-dimethyl- (DMH)	0.12 (15.58)	−2.03 (8.97)	0.028
1,3,5,7-Cyclooctatetraene (COT)	0.22 (0.64)	−0.16 (0.60)	0.037

p-value = significance level oxygen dive compared with air dive. VOCs printed **bold** are methyl alkanes.

than a lower level (Table 4). No significant correlations were found between the remaining VOCs.

After oxygen dives MDA increased from 3.85 (IQR 1.50) mmol l^{-1} to 4.30 (IQR 1.50) mmol l^{-1} whereas after air dives MDA decreased from 4.80 (IQR 1.80) mmol l^{-1} to 3.80 (IQR 0.90) mmol l^{-1}. These changes between pre- and post dive measurements were not significant in either type of dive ($p = 0.28$ for oxygen dives and $p = 0.17$ for air dives) or between both types of dives ($p = 0.27$). Furthermore, excluding the smokers did not lead to significant changes in outcome levels of MDA in both diving groups. Finally, no correlation was found between Δ VOCs and Δ MDA in either type of dive ($0.30 < p < 1.00$ for oxygen diving and $0.42 < p < 0.93$ for air diving).

4. Discussion

The present study shows that a submerged oxygen dive to 9 msw for 1 h results in significant increases in methyl alkanes and other volatile organic compounds (VOCs), which are, however, not necessarily clinically relevant. The oxidative stress the subjects encountered was considered low, based on the non-significant changes in malondialdehyde (MDA). A strong, positive correlation was found between 4-ethyl-5-methyl-nonane and 2,4-dimethylhexane. In contrast, no correlation was found between the other individual VOCs or between VOCs and MDA levels. These findings support our hypothesis that oxygen diving produces a distinguishable VOC breath print, which consists mainly of methyl alkanes.

To our knowledge this is the first study to measure exhaled VOCs before and after submerged, hyperbaric oxygen exposures. All previous studies were performed in a normobaric or dry hyperbaric environment. The present data show that submerged, hyperbaric oxygen exposure generates an increment in methyl alkanes. This is in keeping with the results of Phillips et al. (2003), but in contrast to the results from other studies (Lemaitre et al., 2002; Loiseaux-Meunier et al., 2001; Morita et al., 1986; Suzuki, 1994). Our results suggest that changes in methyl alkanes are a sensitive metabolic biomarker regarding submerged, hyperbaric oxidative stress.

Of the VOCs that differed between oxygen and air dives, half of them were based on methyl alkane. Methyl alkanes are considered to originate from lipid peroxidation of cell membranes (Buszewski et al., 2007; Phillips et al., 2000, 2003). As the lung is the first organ that encounters the high levels of partial oxygen pressure (hyperoxia) it is plausible that alveolar or bronchial cell membranes are the main source of these methyl alkanes (Buszewski et al., 2007). However, we cannot exclude a systemic origin. Of the remaining VOCs only 1,3,5,7-cyclooctatetraene has been reported as a VOC found in human breath (i.e. lung cancer patients) (Peng et al., 2009), but none of these have previously been related to lipid peroxidation. This raises the question whether lipid peroxidation is the only pathophysiological pathway with regard to the early onset of pulmonary oxygen toxicity in the studied group. First of all, a solely adaptive mechanism to hyperbaric hyperoxia could be responsible for the reported changes in VOCs. Indeed, Massaro and Massaro (1974) found an adaptation to hyperoxia in rats after oxygen exposure of 96 h but not after 48 h. However, to our knowledge, no such mechanism has been reported in humans. Therefore, we do not think that adaptation to hyperoxia explains our results, although we cannot exclude such an adaptive mechanism as a confounding factor. Secondly, in this study, because we measured exhaled breath 4 h after oxygen diving, the post-diving sampling time could be of importance. In the previously cited normobaric and hyperbaric oxygen studies alkanes or methyl alkanes were measured up to 2 h after oxygen exposure. Pentane increased immediately after oxygen exposure but reached baseline levels at 60–90 min after termination (Morita et al., 1986). Therefore, in the present study, it is conceivable that oxygen-induced lipid peroxidation had subsided by the time we took the breath samples. This may indicate the involvement of other pathophysiological pathways. Finally, apart from lipid peroxidation, hyperoxia also generates airway inflammation (Clark and Lambertsen, 1971). In airway inflammation many mediator-related pathways appear to be involved that lead to inflammatory responses. As inflammation is thought to be a multistep process this implies that, over time, different pathways may be prominent (Lukacs and Ward, 1996). Airway inflammation itself produces a mix of VOCs containing both lipid peroxidation-based VOCs as well as specific inflammatory VOCs like nitric oxide, nitrate, and nitrogen- and sulphur-containing VOCs (Boots et al., 2012). This hypothesis of airway inflammation is partially supported by the present results. As mentioned above, the lipid peroxidation component is represented by an increase in methyl alkanes. However, to our knowledge, none of the remaining VOCs have been linked to airway inflammation. To gain more insight into the early development of POT, more studies are needed to test this airway inflammation hypothesis. These studies should include multiple post-exposure sample times.

The strengths of this study include its double-blind crossover design and its practical relevance for diving operations as opposed to a dry normobaric or hyperbaric experiment. Nevertheless, some limitations of this study should be addressed. First, exhaled breath was sampled from a vital capacity exhalation and stored in a Tedlar bag before being transported to Tenax filled stainless steel absorption tubes. This sampling method has recently been validated (van der Schee et al., 2012). The exhaled air from a vital

Table 4
Kendall's tau correlation coefficient between significantly changed VOCs with their 95% confidence interval in brackets.

	MOC	MCH	EMN	DMH
MCH	0.04 (−0.37–0.46)			
EMN	−0.09 (−0.37–0.45)	0.33 (−0.19–0.86)		
DMH	−0.13 (−0.58–0.32)	0.31 (−0.14–0.77)	0.73 (0.45–1.00)*	
COT	0.18 (−0.39–0.74)	0.29 (−0.25–0.83)	−0.02 (−0.74–0.70)	0.00 (−0.72–0.72)

* $p < 0.05$. MOC = 2-methyloctacosane; MCH = methyl-cyclohexane; EMN = 4-ethyl-5-methyl-nonane; DMH = 2,4-dimethylhexane; COT = 1,3,5,7-cyclooctatetraene.

capacity manoeuvre contains a mix of both alveolar air and air derived from dead-space. Regarding POT, it is thought that the alveolus is the main inflicted locus making the VOCs derived from this area of special interest. As mixed exhaled air samples are contaminated by nasal or oral VOCs it is recommended to use only alveolar exhaled air and not mixed air as we did (Montuschi et al., 2010).

Secondly, the period of time between storing the exhaled breath in Tenax filled tubes and the measurement of VOCs by GC–MS was up to 2 months. It is reported that a storage time of up to 2 weeks does not influence the diagnostic accuracy by GC–MS (van der Schee et al., 2012), whereas longer storage time bears a potential risk of decomposition of the VOCs (Amann et al., 2012). Therefore, we cannot exclude bias, even though this would have similarly affected all samples in both arms.

5. Conclusion

The present study is the first to measure volatile organic compounds (VOCs) after submerged hyperbaric oxygen exposure. The results show that an oxygen dive to 9 msw for 1 h produces a distinctive VOC breath print, which mainly consists of methyl alkanes. The pathophysiological pathway responsible for this VOC breath print remains to be determined, but could be either a lipid peroxidation-induced pathway, an inflammatory pathway, or both. To gain more insight into the underlying pathophysiology, further studies with oxygen divers are necessary in which different dive depths and dive times are used. Furthermore, multiple post-exposure sample times must be included.

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