Chapter VII

Thesis summary and conclusions.
Main results of investigations described in this thesis

Chapter 2 provides an overview of the available scientific research on the known hazards of oxygen diving. Primarily, we elaborate on the clinical aspects of oxygen toxicity and operational consequences of pulmonary oxygen toxicity (POT) and oxygen toxicity of the central nervous system (CNS-OT). Additionally, current models predicting the chances of POT and CNS-OT occurring and their limitations are discussed.

Chapter 3 presents a randomized double-blind cross-over trial consisting of twelve Navy divers who dove to 9 msw for 1 h with either oxygen (FiO2 1.0, PO2 1.9 ATA) or pressurized air (FiO2 0.21, PO2 0.40 ATA). Pulmonary function tests (PFT), including diffusion capacity of carbon monoxide (DLCO) and nitric oxide (Dlno), which are currently regarded as the gold standard for POT detection did not detect differences between the air and oxygen dives. However, exhaled breath analysis using gas chromatography-mass spectrometry (GC-MS) identified seven volatile organic compounds (VOCs) of interest after oxygen diving: cyclohexane, 2,4-dimethylhexane, 3-methylnonane, 3-[(1,1-dimethylethoxy)methyl]heptane, nonanal, decane and decanal. The intensity of these VOCs increased after the dive, and were 35% higher at 3 h post-dive. In the literature, cyclohexane and nonanal are associated with inflammation, and the remaining methyl alkanes probably originate from lipoperoxidation of alveolar membranes. We conclude that exhaled breath analysis is a more sensitive marker of POT than the current gold standard (PFT) and that VOCs associated with inflammation and lipoperoxidation could be VOCs of interest when assessing POT.

The prospective cohort study described in chapter 4 investigates the effect of daily hyperbaric oxygen therapy (HBOT; 80 min, FiO2 1.0, PO2 2.5 ATA) on exhaled breath in Navy recompression-certified personnel. After 5 days HBOT (Monday to Friday), two rest days and a last HBOT session on the following Monday, eleven VOCs are identified: cyclohexane, hexane, methylcyclohexane, 2,4-dimethylhexane, 3-methyleneheptane, 2,4-dimethylheptane, 3-methylnonane, nonanal, 1-nonanol, decane and 3-methylundecane, which are separated into two groups using principal component analysis. These two groups are similar to those reported in chapter 3: markers of inflammation (principal component (PC) 1: cyclohexane, 1-nonanol and nonanal) and markers of lipoperoxidation of alveolar membranes (PC2: the remaining VOCs). Whereas the intensity of PC1 remained (non-statistically significant) unchanged, the intensity of principal component 2 decreased significantly ($p = 0.001$) to
50.8% 4 h after the first HBOT session, remained between 65% and 101% (mean 82%) on the subsequent days and was still 58% compared to baseline after two days of rest. Perhaps this decrease of intensity can be attributed to the 5 min “air-breaks” in HBOT sessions, which might give the lung enough time to recover from hyperoxic stress. Alternatively, it might be due to the lack of immersion, which has a significant impact on pulmonary function. After all sessions, PFT remained virtually unchanged. Again, we conclude that exhaled breath is a more sensitive marker for obtaining insight into the development of POT. Additionally, as the intensity of the identified VOCs decreased post HBOT as opposed to increased post immersed oxygen dive (chapter 3), we conclude that HBOT (also called ‘dry dives’) cannot be used to determine safe limits for immersed (i.e. ‘wet’) dives.

The field study in chapter 5 entails two scenarios resembling the operational circumstances of the Netherlands Maritime Special Operations Forces (NLMARSOF). The first scenario consisted of a shallow (3 msw) endurance (4 h) closed circuit oxygen rebreather (O2-CCR) dive with divers who were rested. In the second scenario the divers made a 3 h O2-CCR dive to 3 msw as part of a training scenario after five days of vigorous physical exertion, little sleep and very little food. The intensities of three VOCs increased post-dive compared with baseline; cyclohexane ($p = 0.178$, increase 87–433%), 2,4-dimethylhexane ($p = 0.048$, increase 273–461%) and 3-methylnonane ($p = 0.016$, increase 212–415%). These VOCs are associated with inflammation and lipoperoxidation of the alveolar membrane. We conclude that VOCs identified under field conditions are similar to those detected under laboratory conditions, which is important for assessing the practical relevance of the findings in this thesis.

Chapter 6 describes exhaled breath analysis using an electronic nose (eNose) after the dives described in chapter 3. Using principal component analysis, the eNose could distinguish air and oxygen dives optimally 30 min post-dive ($p = 0.003$, AUC 79.9% (61.1–98.6)). Surprisingly, 3 h post-dive, whereas GC-MS detected a 35% increase in the intensities of the identified VOCs, eNose could not distinguish changes in signals accurately ($p = 0.355$, AUC 54.2% (29.8–78.5)). Two-way orthogonal partial least square (O2-PLS) regression demonstrated that the datasets of GC-MS and eNose were associated to each other, and showed the contribution of each sensor array to detection of the identified VOCs. This model had an explained variance ($R^2$) of 0.50, which could be considered moderate. Furthermore, the association between all GC-MS ion fragments and the sensor data was poor, with an $R^2$ of 0.08. We feel these results reflect fundamental differences in the way GC-MS and eNose detect compounds in exhaled breath,
resulting in eNose picking up information that GC-MS does not and vice versa. We conclude that an off-the-shelf eNose platform is currently not accurate enough to detect POT after hyperbaric hyperoxic exposure, but the platform could be modified to include sensors more sensitive to the targeted VOCs in future applications.

Conclusions and future research

Relating the identified exhaled breath markers to POT

The aim of this thesis is to evaluate whether exhaled breath analysis can be used to detect the onset and development of POT after hyperbaric hyperoxic exposure. In the experiments described in chapters 3 to 5 we consistently identified changes in markers of inflammation and lipoperoxidation of phosphatidylcholine using GC-MS. Although different hyperbaric hyperoxic exposures were employed and the participating subjects differed from one another, there is still a risk of bias as the studies were conducted at a single center. However, our data resembles those published previously in a pilot study and recent data of a different research institute (references 16, 29 and 30 of chapter 5) that employed different techniques for capturing and analyzing exhaled breath as well as different subjects and different hyperbaric hyperoxic exposures. Although this cannot be considered enough evidence to validate our findings, we feel this substantiates our results.

It should be noted that the hyperoxic exposures described in this thesis did not induce clinical symptoms of POT, such as dyspnea, coughing or retrosternal pain. Longer and deeper dives with pure oxygen, as used in the past to determine the current gold standard, could provide more valuable data on the VOCs emitted in POT. Failure to show an association between these molecular findings and clinical symptoms of POT remains an important limitation of these studies, which should be addressed in future studies. However, exposing subjects to longer and deeper divers would increase the risk for CNS-OT as described in chapter 2. With our modern perspective on medical ethical considerations it is unlikely that these experiments will be conducted in the near future and ‘submaximal’ hyperbaric hyperoxic, such as those used in this thesis and in other studies, are likely to remain the norm.

This raises the issue of whether we induced ‘severely enough’ POT to accurately detect its onset and development. More so, the PFT results, which are still regarded as the gold standard
for detecting POT, were almost unchanged after the hyperbaric hyperoxic exposures used in this study. Because changes in the constituents of exhaled breath analysis were detected using GC-MS and eNose, exhaled breath analysis may have potential for POT detection at an early stage. However, it is still difficult to pinpoint a single exhaled molecule that marks the onset of POT. Perhaps this can be attributed to the method GC-MS data is analyzed. A single breath yields between 10,000 and 25,000 potentially relevant ‘spikes’ in intensity, many of which should be regarded as background noise. This modality should be regarded as ‘big data’. A commonly used technique to reduce the amount of data is a signal-to-noise filter of 100 to 1, assuming that intensities of compounds of interest are a 100-fold larger than background noise. While this is a scientifically accepted method for handling GC-MS data and yielded promising results in our studies, it could also lead to potential exclusion of relevant compounds that have an intensity just below the 100-to-1 threshold. Additionally, the lipoperoxidation of phosphatidylcholine can lead to different methyl alkanes, depending on the site where the oleate tail breaks and the fragments methylate. For example, 2,4-dimethylhexane and 3-methylheptane can result from the same lipoperoxidation process and are difficult to distinguish from each other using GC-MS. Searching for a single molecule as a marker of POT may therefore be futile. We recommend that future research should focus on detecting methyl alkanes as a group, not as individual molecules.

Is eNose technology the future of exhaled breath research in regard to POT?

While both eNose technology and GC-MS can analyze exhaled breath, these techniques rely on different principles. As described in chapter 6, eNose provides additional information that GC-MS does not and vice versa. Most importantly, eNose detects the characteristics of groups of molecules instead of identifying every single molecule. Therefore, eNose can overcome a major limitation, i.e. reliance on the detection of single molecules, described in the previous section. Additionally, very small or highly volatile components that cannot be detected using GC-MS can induce sensor data in eNose. However, eNose sensor data is difficult to link to pathophysiological processes, as sensor data is not equivalent to molecular identification. This is discussed in chapter 6.

Using O2-PLS regression, the correlation found between VOCs of interest and sensor data, as well as sensor data to ion fragments, was moderate at best. While this seems disappointing, to our knowledge this is the first study to attempt associating the results of the two
exhaled breath modalities with each other. Additionally, this information provides us with an opportunity to modify existing eNose platforms to equip them with sensors that can better detect the characteristics of VOCs of interest. Future iterations of an eNose with sensor arrays designed to provide more POT-relevant data could be tested and further improved using GC-MS data in combination with O2-PLS regression. This approach could reduce the time and cost of developing a low-cost and highly accurate diagnostic device to assess POT on a large scale.

However, even if an optimal eNose platform is developed, many important questions remain to be answered. Firstly, all studies of exhaled breath analysis have compared pre versus post exposure data. Currently, there are no known ‘normal’ or ‘baseline’ values, which makes assessment of POT purely based on GC-MS or eNose data in individual cases difficult. We feel that these technological advances cannot replace thorough history-taking and physical examinations by hyperbaric or diving medical physicians. We hope however, that such a device will help detect ‘subclinical’ or ‘developing’ POT before it becomes abundantly clear at later stages. Furthermore, we have not covered differences in individual susceptibility to POT. As with CNS-OT, it stands to reason that various factors affect one’s risk of developing POT. Which factors these are, and how they can be detected, remains to be assessed in future research. Lastly, different hyperbaric hyperoxic exposures, both with respect to PO2 and time, will be required to establish a dose-response curve or equation that can ultimately be used to replace the current standard.

**Operational consequences**

Depending on the task at hand, Navy divers can employ dive systems that expose them to high partial pressures of oxygen for long periods of time. In this chapter, we described exposures to a PO2 of 1.9 ATA for 1 h and 1.3 ATA for 4 h. The latter resulted in a substantially larger increase in the intensity of VOCs of interest (roughly 10 times greater), but did not induce any clinical symptoms. Although studies with longer or higher PO2 exposure would be insightful from an academic perspective, these dive profiles should also be assessed if they are realistic and relevant to military operations.

As outlined in chapter 4, the exhaled breath profile of a subject in a recompression chamber shows changes in VOCs similar to those observed after immersed (‘wet’) dives, but the inten-
sities of the VOCs were inversed, i.e. emission decreased after dry diving but increased after immersed diving. This confirms earlier hypotheses that results collected from dry dives cannot be extrapolated to in water diving. This is an important consideration for future studies of oxygen toxicity of in water diving, as research entailing wet dives requires more resources and safety precautions that those of dry dives.

Furthermore, because the subjects in our studies have performed only ‘single dives’, we cannot be sure whether the development of POT is affected by multiple dives in a short timeframe. It stands to reason that repetitive exposures to a high \( \text{PO}_2 \) will accelerate the development of POT, but to what extend and how much time is required to recover is currently unknown.

Diving for extended periods impacts the physical performance of an individual during and especially after the dive. Factors such as dehydration, hypothermia and just plain fatigue can incapacitate an individual after straining diving operations. As military diving is always part of an operation, for instance mine clearance diving or gaining access to a hostile environment unseen, as described in the preface, these contextual factors of oxygen diving should be taken into account. To what extend oxygen diving affects performance is currently not well defined, and we consider this a primary reason for continuing this research, the results of which have important implications for military diving.