Oxygen concentration in inhaled gas and nitric oxide concentration in exhaled gas.

Is nitric oxide concentration in exhaled gas a useful marker for exposure to hyperoxia?

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1. Scientific environment

The work presented in this PhD thesis was carried out during the years 2008 to 2013 emerged from the Institute of Medicine and Dentistry, University of Bergen, Norway. Statoil, Gassco and ExxonMobil supported the PhD scholarship through the Competence program – Diving 2007-2011.

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3. Summary of thesis

BACKGROUND: Exposure to partial pressures of oxygen (PO_2) higher than 50 kPa may result in toxic effects on the lungs with reduced vital capacity, maximal expiratory flow rates and diffusion capacity. The risk of developing pulmonary oxygen toxicity is present during diving and hyperbaric oxygen therapy. The traditional lung function tests are not sufficiently sensitive, and new markers of early development of oxygen toxicity are required. Nitric oxide (NO) in exhaled gas is a marker of some inflammatory processes in the lungs and the production of NO is influenced by the PO_2. The fraction of NO in exhaled gas (FE_{NO}) is reduced by 30% after exposure to hypobaric hypoxia at altitude above 4500meter. NO from the alveolar and bronchial compartments of the lung contribute to FE_{NO}. Changes in NO concentration at the alveolar level may have effects on pulmonary blood flow and gas exchange, and thereby contribute to the reduction in diffusion capacity. A reduction in bronchial NO flux is associated with induced bronchoconstriction, while increased alveolar NO concentration is associated with increased alveolar dead space.

AIMS:

The overall aim in the present thesis was to investigate if FE_{NO} is a useful marker to predict pulmonary oxygen toxicity. This was investigated through three separated studies that aimed at:

1. To study the dose-response relationship between oxygen exposure and FE_{NO} during and after hypoxia and hyperoxia.
2. To investigate the alveolar and bronchial contributions in FE_{NO} after exposure to hyperoxia.
3. To investigate the relationship between FE_{NO} and lung function.

DESIGN: An open randomised crossover design and a non-randomized design.

METHODS: 73 healthy non-smoking subjects between the ages of 20-40 years and a smaller group of 12 patients between 35-68 years undergoing hyperbaric oxygen therapy were included. FE_{NO} was measured before, during and after exposure to hypobaric hypoxia (PO_2 = 15 kPa), hyperbaric hyperoxia (PO_2 = 240 kPa), normobaric hyperoxia (PO_2 = 100 kPa) and when breathing ambient air (PO_2 = 21 kPa), all for 90 min. Dynamic and static lung volumes, maximal

expiratory flow rates, distribution of ventilation and diffusion capacity (D_LCO) were measured before and after exposure to normobaric hyperoxia.

RESULTS: The concentration of NO in exhaled gas was reduced by 20-30% after exposure to hyperoxia at PO_2 of 100-240 kPa, but no significant change was found after exposure to hypoxia when corrected for altitude effects. The bronchial contribution to NO in exhaled gas was reduced after exposure to hyperoxia, whereas the alveolar NO concentration was unchanged. There was no association between the change in FE_{NO} and the changes in lung function after exposure to normobaric hyperoxia.

CONCLUSIONS:

1. The partial pressure of NO in exhaled gas was unchanged upon arrival at moderate altitude after correcting for gas density effects. There might be a dose-response relationship between PO_2 and the change in FE_{NO}, where subjects exposed to the highest dose of oxygen showed the greatest reduction in FE_{NO}.

2. There was a significant reduction in bronchial contribution to NO in exhaled gas after hyperoxia, but no change in the alveolar NO concentration.

3. The reduction in FE_{NO} by 20% after exposure to hyperoxia at PO_2 of 100 kPa for 90 min was not associated with the changes in lung function.

FE_{NO} was not confirmed as a direct marker of pulmonary oxygen toxicity.

KEY WORDS: Altitude; diving; exhaled nitric oxide; hyperbaric hyperoxia; hypobaric hypoxia; normobaric hyperoxia; hyperbaric oxygen therapy; lung function.

4. Abbreviations

ATS/ERS	American Thoracic Society /European Respiratory Society
C_ANO	Alveolar nitric oxide concentration
CV	Closing volume
D_LCO	Diffusion capacity for carbon monoxide
FE_{NO}	Fraction of nitric oxide in exhaled gas
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
FRC	Functional residual capacity
HBO	Hyperbaric oxygen exposure
HUH	Haukeland University Hospital
iNOS	Inducible NO synthase
$J_{aw}NO$	Bronchial nitric oxide flux
NBO	Normobaric oxygen exposure
NOS	Nitric oxide synthase
NSSS	Norwegian School of Sport Sciences
m	meter
Ppb	Parts per billion
PE_{NO}	Partial pressure of nitric oxide in exhaled gas
PE_{NOcorr}	Nitric oxide in exhaled gas corrected for gas density effects
PO_2	Partial pressure of oxygen
RV	Residual volume
TLC	Total lung capacity
UIB	University of Bergen
UPTD	Unit pulmonary toxic dose
VC	Vital capacity

5. List of publications

This thesis is based on the following papers, which will be referred to as paper I - III in the text.

Paper I

Exhaled nitric oxide concentration upon acute exposure to moderate altitude.

Caspersen C, Stang J, Thorsen E, Stensrud T. Scand J Med Sci Sports 2013; 23:102-107.

Paper II

Bronchial nitric oxide flux and alveolar nitric oxide concentration after exposure to hyperoxia.
Caspersen C, Stensrud T, Thorsen E. Aviat Space Environ Med 2011; 82:946-50.

Paper III

Exhaled nitric oxide and lung function after moderate normobaric hyperoxic exposure. Caspersen C, Stensrud T, Storebø M, Thorsen E. Undersea Hyperb Med 2013; 40:7-13.

6. Table of content

7. Introduction

7.1 Nitric oxide

7.1.1 The history of nitric oxide

Nitric monoxide, known as nitric oxide (NO) in this thesis, is a simple gas composed of nitrogen and oxygen atoms. Nearly 30 years ago, NO was discovered as one of the smallest biologically active messenger molecules in the cardiovascular and nervous system. Before that time, NO was only believed to be an air pollutant with no function in human biology. Furchgott and Zawadzki discovered endothelium derived relaxing factor in 1980 (1). In 1987, two research groups independently identified NO as endothelium derived relaxing factor (2,3). Palmer et al. (3) discovered that NO production was dependent on L-arginine. In 1991 Gustafsson et al. (4) reported that endogenous NO in exhaled breath was present in humans and in animals. Shortly after, the fraction of NO in exhaled gas (FE_{NO}) was found to be increased in patients with asthma (5). NO was selected the "Molecule of the year" in 1992 (6) and was the subject of the Nobel Prize winning research in 1998.

NO is a signaling molecule, a free radical with a short half-life of ~5 sec that diffuses freely across membranes (7) and has low solubility in water (0.0058 grams per 100 gram water at 1 atmosphere and 25ºC) (8). NO is an essential mediator in inflammatory responses and is increased in eosinophilic airway inflammation (9). Subjects with asthma and atopy have higher levels of FE_{NO} compared to non-atopic subjects. The main contributor to this increase is by inducible NO synthase (iNOS). In addition, NO is a vasodilator that regulates pulmonary vascular tone and a signaling substance in the central nervous system (4,10).

7.1.2 The biology of nitric oxide

NO is shown to have different functions in the human body; both pathological and biological. Septic shock, hypertension, carcinogenesis and atherosclerosis are examples of pathological conditions where NO are involved (11), whereas cardiac contractility, peristalsis, smooth muscle relaxation are functions included in biological processes (12).

NO is a highly reactive radical generated from a five-electron oxidation of a guanidino nitrogen from L-arginine into L-citrulline by the enzyme known as NOS (13). In addition, flavin adenine dinucleotide, nicotinamide adenine dinuecleotide phosphate, flavin mononucletide and tetrahydrobiopterin are essential cofactors necessary for the NOS isoforms to function in general (10) (Figure 1). NO rapidly reacts with oxygen to form nitrites, superoxide to form peroxynitrite and oxyhaemoglobin to form nitrate and methaemoglobin (14). *In vitro* experiments have suggested that N^G-monomethyl L-arginine is a competitive inhibitor of NOS (15,16). The activity of NO depends on several local factors such as the amount and activity of the enzymes responsible for producing NO, its rate of uptake by antioxidant molecules and oxidant stress (17). All isoenzymes catalyzes the same reaction with use of the same substrate and cofactors.

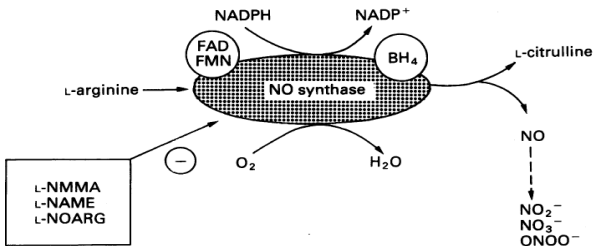

Figure 1: *Schematic representation of nitric oxide (NO) synthase involves conversion of L-arginine to L-citrulline and the cofactors required to for enzyme activity and inhibition; flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucletide (FMN), calcium, calmodulin and tetrahydrobiopterin (BH_4), N^G-monomethyl L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NOARG). Reprinted by permission from Barnes PJ. Thorax 1993; 48:1034-43 (10).*

In the human lung, three isoforms of NOS are identified; the neuronal, endothelial and inducible form. Neuronal NOS is predominantly expressed in neuronal tissue (18) and mainly in postganglionic parasympathetic neurons in the airways (19). This source of NO contributes doubtfully to NO in exhaled air due to the distance from the airway lumen. Endothelial NOS is involved in the control of bronchial and pulmonary blood flow (20). It is unlikely that endothelial NOS contribute considerably to FE_{NO} due to the fact that it generates 1000-fold less NO than iNOS. Neuronal and endothelial NOS are constitutive, calcium/calmodulin dependent and releases low amount of NO for a short period in response to the stimuli that is registered by the

receptors. iNOS is calcium independent and generates NO in great amount for extended periods after exposure to certain cytokines (21). iNOS is mainly expressed in epithelial cells, macrophages and T lymphocytes (11), but also in vascular smooth muscle cells, neutrophils and fibroblasts (12,22). Lane et al. (23) reported that the expression of iNOS correlates with NO in exhaled gas and it is the most important determinant of NO concentration in exhaled breath compared to the other isoforms of NOS in airway epithelium. Batra et al. (24) found that iNOS was distinctly upregulated in the airway epithelium in subjects with asthma.

7.1.3 Nitric oxide in the airways

In the airways, NO is produced by several cell types such as epithelial cells (25), airway nerves (26), vascular endothelial cells (2) and inflammatory cells (18). The last decade monitoring inflammation of the small airways has increased and recent research has showed that NO is produced in conductive airways and in the lower respiratory tract (27-29). NO in exhaled gas measured at multiple flow rates gives information about the distribution of NO in the airways and in the lungs (30). One source is the alveolar NO concentration (C_ANO), where steady state is reached. The second is the bronchial source, where exhaled NO diffuses from the airway wall known as bronchial NO flux ($J_{aw}NO$). It is possible to model NO formation in the lung, which can be simplified in a two-compartment model comprising the airway compartment and the gas exchange compartment. Several models can be used based on analysis of the relationship between the inverse of expiratory flow rate and FE_{NO} (31,32). There are minor differences between the models used to calculate the flow-independent parameters. Most frequently used is a linear model by Tsoukias and George (33), which gives information about C_ANO and $J_{aw}NO$. The non-linear model by Högman and Meriläinen provide additional flow-independent parameters such as airway wall concentrations of NO and airway diffusing capacity and is shown to be more accurate than the linear models (34). This mathematical model uses a set of three different exhalation flow rates from low, medium and high or 10 $mL \cdot s^{-1}$, 100 $mL \cdot s^{-1}$ and 300-500 $mL \cdot s^{-1}$, respectively.

7.1.4 Measurement of FE_{NO}

Mutual guidelines are developed from the American Thoracic Society and European Respiratory Society (ATS/ERS) on how to perform exhaled NO measurements (35). Recommended standardized procedures are necessary due to technical factors affecting exhaled NO such as; expiratory flow rate, breath-hold, exhalation time, inspiration maneuver, oral pressure, ambient NO and time of day for sampling. The recommended expiratory flow rate of 50 $mL \cdot s^{-1}$ is acceptable for both adult and children. FE_{NO} is expressed in parts per billion (ppb), which is equal to nanoliters per liter. The measurement of FE_{NO} is a simple and safe non-invasive method to measure inflammation in the airways during single-breath exhalation in humans (35).

Three methods can be used for direct measurements of exhaled NO; laser spectroscopic, electrochemical sensing and chemiluminescence (36). Two different techniques can be used to measure FE_{NO} with chemiluminescence. The first is a single-breath online technique, where the result is obtained immediately. The online measurement is performed during a single flow-controlled exhalation against a resistance. First the subject exhales slowly, then inhales NO-free air to total lung capacity (TLC) and succeeds instantly by full expiration with a constant flow rate via the mouthpiece. The method is based on a photochemical reaction between NO and ozone generated in the analyser, which leads to the product nitrites. When nitrites returns to its basal levels it emits a photon and total number of photons produced is proportional to the NO concentration in the exhaled gas (37). The test procedure has few technical challenges. It is highly sensitive and regarded as the gold standard for clinical measurements of FE_{NO}. The exhaled gas is immediately analysed and the method makes it possible to carry out repetitive measurements without affecting NO formation. However, the chemiluminescence analyser is expensive and not easy to move around. FE_{NO} measurements are accepted as a complementary tool, in addition to other lung function tests, in the diagnosis and treatment of pulmonary diseases (38).

The second technique is the offline NO measurement where a portable hand-held device is used to collect exhaled gas into impermeable bags. The same breathing technique as for single-breath online measurements is conducted. The gas sample is not analysed directly, but sometime after when returned to the laboratory. The hand-held device makes it possible to measure FE_{NO} outside the laboratory. The sampling gas is stable up to 48 hours in a Mylar® bag, but preferred to be analysed within 12 hours after collection (11).

In the literature, partial pressure of NO in exhaled gas (PE_{NO}) is reported with different units. Therefore, Hemmingsson et al. (39) suggested that exhaled NO concentration should at all times be reported as partial pressure and not as volume fraction to be comparable at different altitude.

7.1.5 Factors affecting the measurments of FE_{NO}

FE_{NO} is a biomarker influenced by constitutional, environmental and pathological factors that may affect exhaled NO concentration. This should be taken into account when evaluating FE_{NO}. The most essential of these are atopy, gender, age and cigarette smoking (11). There are contradictory reports concerning these factors. Table 1 includes an overview of different effects related to FE_{NO}.

FE_{NO} is increased in several inflammatory processes in the lung such as atopic asthma and allergic rhinitis (40). FE_{NO} may serve as a non-invasive marker in some type of airways inflammation, particularly eosinophilic inflammation (41). Uncertain results are shown in chronic obstructive pulmonary disease, which is associated with neutrophilic inflammation (42,43).

Several studies have reported lower FE_{NO} in women compared to men with gender related differences by 23-53% (44-46). This was also confirmed in the study by Olivier et al. (31). Therefore, they suggested that reference values for FE_{NO} should be gender based. However, in a large population study of 2200 adults no gender difference was found, but there was a positive association with height and age (47). A positive correlation was also found between FE_{NO} and weight in healthy subjects (48,49), but the results are inconsistent concerning the effect of body mass index or weight.

There are different results regarding FE_{NO} and age in adults. Olin et al. (47) reported 40% higher FE_{NO} in subjects > 60 years compared to subjects < 30 years. On the other hand, Ekroos et al. (50) found no correlation between FE_{NO} and age. This could be explained by the small age range of 21-48 years.

In current smokers FE_{NO} is decreased with 40-60% and may correlate with the cumulative lifetime cigarette consumption (45,47,51). In previous smokers a small decrease in FE_{NO} of around 10% has been reported (45,52), whereas Olin et al. (47) found no effect.

Other sources that may influence FE_{NO} is air pollution, upcoming respiratory infections and oral bacterial flora (19). There are also conflicting results concerning the effect of exercise (53-55), menstrual cycle and pregnancy (56,57). It is recommended to avoid great amount of nitrate-rich food one day before testing. FE_{NO} can be increased by > 150% after ingestion of nitrate-rich food and the effect may last up to 15 hours (58). The spirometry maneuver may influence and briefly decrease $FE_{NO.}$ Therefore, it is recommended to measure lung function after NO sampling (35). However, a recent study found no effect of spirometry on FE_{NO} in healthy subjects (59).

Table 1: *Physiological, pathophysiological conditions and habits of different effects related to the fraction of nitric oxide in exhaled gas (FE_{NO}).*

Decreasing FE_{NO}	**Increasing FE_{NO}**	**Uncertain effect on FE_{NO}**
Acute respiratory distress syndrome	Age	Age in adults
Alcohol ingestion	Air pollution	Body mass index
Body temperature reduction	Allergic rhinitis	Bronchiectasis
Bronchoconstriction	Alveolitis	Caffeine
Bronchial provocation	Asthma	Chronic obstructive pulmonary disease
Cystic fibrosis	Atopic subjects	Diurnal variation
Heart failure	Breath-hold	Gender
Human immunodeficiency virus	Bronchiectasis	Menstrual cycle
Hypertensive subjects	Eosinophilic bronchitis	Pregnancy
Hypertension	Fibrosing alveolitis	Physical exercise
Hypoxia	Height	Spirometric manoeuvres Sputum induction
Influenza virus	IgE sensitisation	Systemic sclerosis
Inhaled corticosteroids	Influenza vaccination	Weight
Mouth washing	Lung cancer	
Primary ciliary dyskinesia	Nasal polyposis	
Pulmonary hypertension	Nitrate enriched diet Pulmonary sarcoidosis	
Primary cilia dyskinesia	Rhinovirus	
Pneumonia	Respiratory infections	
Smoking	Tuberculosis	
Sputum induction	Upper respiratory tract infection	
Syncytial virus	Ulcerative colitis	
	Viral infection	

7.1.6 FE_{NO} and hypoxia

The partial pressure of oxygen (PO_2) and the gas density are proportionately reduced with the reduction in ambient pressure. In high altitude the availability of oxygen is decreased, which will lead to less oxygen in the blood flow and decreased oxygen saturation of haemoglobin. Cardiac output need to increase to improve oxygen delivery to the tissue.

FE_{NO} has been shown to be decreased by ~30% at altitude above 4500meter (m) (27,60). Hemmingsson et al. (39) reported that FE_{NO} is influenced by altitude conditions related to reduced gas density and increased expiratory flow rate. Therefore, when measuring FE_{NO} at altitude it is important to determine how expired flow varies and make accurate offline corrections to convert FE_{NO} to PE_{NO}. To compare FE_{NO} in different environments one unit is necessary. This unit has to be independent of the surroundings such as molar concentration or partial pressure. Hemmingsson et al. (39) showed how the reduction in PE_{NO} depends on altitude performed in an altitude chamber at ambient pressures of 540-899 hPa (410-640 mmHg) corresponding to 1000-5000m. In field studies, similar reductions in PE_{NO} have been demonstrated including physical effort by tracking or climbing upon arrival at 4200 and 4559m (60,61). Recently, two studies reported a gradual decrease in PE_{NO} during prolonged exposure to altitude (39,62). See table 2 for an overview of studies.

Table 2: *An overview of studies investigating the fraction of nitric oxide in exhaled gas (FE_{NO}) and the partial pressure of nitric oxide in exhaled gas corrected for altitude conditions (PE_{NO}) in humans after exposure to hypobaric hypoxia. (Meter above sea level = masl).*

Ref	Author	Year	Time	Masl	Δ FE_{NO}	Δ PE_{NO}
(61)	Brown et al.	2006	75 min 0 hr	2800 4200		↓ 10%* ↓ 21%*
(63)	Donnelly et al.	2011	12 hrs 7 days 20 days	1336 2800 5050	↓ 9% ↑ 20% ↓ 15%**	↓ 21% ↓ 13% ↓ 52%**
(60)	Duplain et al.	2000	12 hrs 24 hrs 48 hrs	4559 4559 4559		↓ 20% ↓ 14% ↑ 3%
(64)	Hemmingsson et al.	2009	10 min	3100 5000		↓ 15% ↓ 33%
(62)	Vinnikov et al.	2011	1 day 3 days 21 days	3800 3800 3800	↓ 48% ↓ 21% ↓ 4%	↑ 2% ↓ 17%** ↓ 30%**

* = $p < 0.01$, ** = $p < 0.001$.

7.1.7 FE_{NO} and hyperoxia

There are conflicting results regarding studies in humans exposed to normobaric hyperoxia (NBO). There was no change in FE_{NO} after breathing 100% or 40% O_2 for 90 min in healthy subjects (65). Tsuchiya et al. (66) found a decrease in FE_{NO} after breathing 100% O_2 for 50 min, but no change after breathing 40% O_2 for 50 min in subjects that were mechanically ventilated during anaesthesia. On the contrary, Schmetterer et al. (67) reported an increase of 25% in FE_{NO} when breathing 100% O_2. FE_{NO} is reduced by ~30-70% after exposure to hyperbaric hyperoxia to a PO_2 of 240 kPa for 90 min (65,68,69). The reduction in FE_{NO} persists for more than 4 hours, but is apparently returned to normal within 24 hours (68). A reduction in FE_{NO} of 55-63% has been demonstrated in healthy divers exposed to a PO_2 of 203 kPa for 6-8 hours in a hyperbaric chamber (70) (Table 3).

Table 3: *An overview of previous studies investigating the change in the fraction of nitric oxide in exhaled gas (Δ FE_{NO}) in humans after exposure to normobaric and hyperbaric hyperoxia (mean ± SD).*

Ref	Author	Year	Dose (O_2% x min)	Δ FE_{NO}
(71)	MacInnis et al.	2012	100 x 15	↑ 38%*
(65)	Puthucheary et al.	2006	40 x 90	↓ 13%
(67)	Schmetterer et al.	1997	100 x 10	↑ 25%
(72)	Schmetterer et al.	1997	100 x 15	↑ 38%*
(66)	Tsuchiya et al.	2000	40 x 50	No change
(70)	Fothergill & Gertner	2009	203 x 360 203 x 480	↓ 55%** ↓ 63%**
(68)	Kjelkenes et al.	2009	240 x 90	↓ 30 ± 22%*
(65)	Puthucheary et al.	2006	240 x 90	↓ 71%**
(69)	Taraldsøy et al.	2007	240 x 90 (day 1) 240 x 90 (day 25)	↓ 33 ± 8%** ↓ 41 ± 9%**

* = $p < 0.01$, ** $p = < 0.001$.

7.1.8 FE_{NO} and altitude conditions

In this thesis the hypoxic exposure is included, in addition to hyperoxia, to establish the dose-response relationship between oxygen exposure ((PO_2 – 21 kPa) * time of exposure in minutes) and the change in FE_{NO}. Lately, it has become more common to measure FE_{NO} in athletes during training programs at altitude. Elite athletes exercise and stay at altitudes of 2000-3000m for periods of 2-3 weeks to benefit from higher haemoglobin concentration. The prevalence of asthma and bronchial hyper-responsiveness among athletes is high, especially within endurance sports (73-75). Prolonged intensive training in unfavorable environment may cause this during their sports career (76-78). Asthma developed from childhood is associated with increased FE_{NO} and eosinophilic airway inflammation (79). This should be separated from "sports asthma" starting in adulthood where FE_{NO} is decreased and eosinophilic inflammation is not present (74,80).

The high amount of endurance training in combination with the challenge to control the training intensity at altitude may exacerbate bronchial hyper-responsiveness and bronchial inflammation both in asthmatic and non-asthmatic athletes. However, there was no change in exercise-induced bronchoconstriction in asthmatic subjects upon exposure to an altitude of 2500m (81). At altitude there is lower temperature and water content of air compared with sea level, and the ventilatory demand is higher. Increasing altitude will lead to a reduction in PO_2 and gas density, which will influence FE_{NO} together with increased expiratory flow rate. This needs to be taken into account for correct estimation of PE_{NO}. This is important for athletes who want to monitor airway function at different altitudes over time.

7.2 Oxygen tocixity

Oxygen toxicity is a concern during neonatal care, nitrox diving and hyperbaric oxygen (HBO) therapy. The cumulative oxygen dose resulting in toxicity is determined by atmospheric pressure, duration, the fraction of inspired oxygen and the susceptibility of the subject (82,83). Oxygen toxicity in the central nervous system may occur when the exposure time is short and the concentration of oxygen exposure is high > 300 kPa. This is known as the Paul Bert effect and may occur during HBO therapy (83). Pulmonary oxygen toxicity may occur after prolonged exposure to increased oxygen levels at normal barometric pressure at 40-50 kPa and higher. This is referred to as the Lorraine Smith effect or the low pressure oxygen poisoning. Breathing oxygen for more than 10 hours at a PO_2 of 100 kPa may lead to the first signs of toxicity (83).

Pulmonary oxygen toxicity is associated with inflammatory responses in the airways and in the alveoli causing alveolar epithelial and endothelial dysfunction and, finally, pulmonary edema (84,85). Exposure to hyperoxia is a main contributor to the long-term effects of diving on the lung, which are characterized by reduction in airways conductance and diffusion capacity (86). NO as an exhaled breath marker is related with inflammatory processes in the lung and recently found to be markedly affected by oxygen exposures (69-71). Previous studies have reported a reduction in FE_{NO} by 30-70% at a PO_2 of 203-240 kPa (69,70). The mechanisms behind the changes in expired NO concentration with oxygen exposure are not fully understood. However,

there is increasing evidence to suggest that there is a link between pulmonary oxygen susceptibility and FE_{NO}.

The primary mechanism for pulmonary oxygen toxicity is assumed to be increased production of reactive oxygen species (ROS). Inflammatory responses are secondary to cell and tissue damage caused by ROS and peroxynitrite. Endothelial integrity is necessary for endothelial NO production and vasoregulation. In addition, NO dependent neurogenic mechanisms are involved in development of pulmonary edema and seizures associated with exposure to hyperoxia. An up regulation of NOS may be associated with these serious consequences of oxygen toxicity. In healthy subjects the origin of NO in exhaled gas is mainly from the bronchial epithelial cells and synthesized by iNOS.

Clark and Lambertsen (87) established a predictive graphic model involving time of exposure, PO_2, and toxicity, expressed as a decrease in vital capacity (VC). They found a dose-dependent decrease in VC after exposure to a PO_2 higher than 50 kPa, which is the traditional sign of oxygen toxicity (88,89). 50 kPa is corresponding to a dive at 15m. Based on the changes in VC, the unit pulmonary toxic dose (UPTD) was established to give guidelines for tolerable oxygen exposure in operational diving and in HBO treatment protocols (90). The degree of pulmonary oxygen toxicity produced by breathing 100% oxygen continuously at a pressure of 1 atmosphere for 1 min equals one UPTD. The pulmonary oxygen tolerance curve by Clark and Lambertsen indicate a reduction in VC by 2-4% after about 12-16 hours of inspired oxygen partial pressure at 1 atmosphere (87).

The use of VC as a marker of oxygen toxicity may have limitations since FE_{NO}, the diffusion capacity of the lungs for carbon monoxide (D_LCO) and maximal expiratory flow rates can be reduced before measurable reduction in VC occurs (91). A single-breath technique showed a reduction in diffusion capacity by 30% after 48 hours exposure to breathing 98% oxygen (92), whereas Rosenberg & MacLean reported no change in D_LCO after 3 hours exposure to 100% oxygen (93). D_LCO was decreased after deep saturation dives (3.1-4.6 MPa) (94,95) and some studies has shown an increase in static lung volumes, forced vital capacity (FVC) and forced expiratory flow in one second (FEV_1) (96,97).

8. OBJECTIVES

The overall aim in the present thesis was to investigate if nitric oxide in exhaled gas is a useful marker to predict pulmonary oxygen toxicity. This was investigated through three separated studies that aimed at:

1. To study the dose-response relationship between oxygen exposure and FE_{NO} during and after exposure to hypoxia and hyperoxia in healthy humans.

2. To investigate the alveolar and bronchial contributions in FE_{NO} after exposure to normobaric and hyperbaric hyperoxia in humans.

3. To investigate the relationship between FE_{NO} and lung function after exposure to normobaric hyperoxia in healthy humans.

9. SUBJECTS AND METHODS

9.1 Ethics

The present studies were performed according to the ethical guidelines defined by the World Medical Association's Declaration of Helsinki and approved by the Regional Committee for Medical Research Ethics, Bergen, Norway. All subjects signed a written informed consent prior to all testing.

9.2 Subjects

The subjects included several groups of students recruited from the Norwegian School of Sport Sciences (NSSS) and the University of Bergen (UIB), and patients from Haukeland University Hospital (HUH). An overview of the different groups and demographics is given in Table 4. The subjects had no heart or lung disease and were non-smoking. The healthy subjects did regular physical exercise at least two times a week. Subjects were excluded if there was a history of disease within the last 14 days, current use of tobacco or asthma. One subject was excluded due to disease during testing and did not perform the control exposure in paper I.

Table 4: *An overview of the demographics in subject's exposed to hypobaric hypoxia for 5 (HH_5) and 90 min (HH_{90}), hyperbaric oxygen (HBO) therapy, normobaric hyperoxia (NBO) and the control group breathing ambient air (AA) (mean ± SD).*

Paper	Exposure	N	Age (yr)	Height (cm)	Body mass (kg)
Paper I	HH_5	9	29 ± 9	166 ± 7	63 ± 9
	HH_{90}	20	26 ± 3	176 ± 10	73 ± 14
Paper II	HBO	12	50 ± 11	174 ± 8	78 ± 16
	NBO	20	27± 4	172 ± 9	67 ± 10
	AA	6	28 ± 4	172 ± 10	71 ± 9
Paper III	NBO	18	24 ± 2	176 ± 9	67 ± 11

9.3 Study design

The studies presented in this thesis are based on an open randomised crossover design in paper I and III, and a non-randomized design in paper II. The recruitment process was mainly carried out by a brief oral presentation concerning the project. In addition, subjects were recruited by telephone, e-mail and word to mouth. The main investigator carried out all recruitment.

Paper I

The data was collected at the respiratory laboratory at NSSS from March to May in 2009 and in October 2010. Twenty healthy subjects recruited from NSSS were exposed to hypobaric hypoxia in an altitude chamber (Norwegian Underwater Technique A/S, Haugesund, Norway) at an ambient pressure of 728 hPa (546 mmHg, PO_2 = 15 kPa) corresponding to 2800m for 90 min (Figure 2). The laboratory was located close to sea level at an altitude of 180m. Decompression to 2800m and recompression back to sea level took ~5 min each. FE_{NO} was measured offline and online ~15 min before decompression and ~15 min after recompression. At altitude FE_{NO} was

measured offline at 30, 60 and 90 min. FVC and FEV_1 was measured before and after exposure outside the altitude chamber (Masterscreen; Erich Jeager® GmbH & Co KG, Würzburg, Germany). The healthy subjects functioned as their own control breathing ambient air and were tested at sea level with the same protocol on a different day. In addition, a smaller group of nine healthy subjects performed the same protocol as described earlier to study the immediate effect in FE_{NO} when arriving at altitude. FE_{NO} was measured before, immediately after arrival at altitude for 5 min and after exposure. All exposures were performed at the same time of the day between 9 and 12 am, at least one hour after breakfast.

Figure 2: *The low pressure chamber at the Norwegian School of Sport Sciences, Oslo, Norway.*

Paper II

The data collection was performed at the respiratory laboratory at HUH between February and April and at NSSS December 2010. Twelve patients recruited from HUH were exposed to HBO therapy daily for 90 min in a monoplace hyperbaric chamber for four weeks (figure 3). The

chamber was compressed to a pressure of 240 kPa within 10-15 min. The oxygen exposure was in three intervals of 30 min interrupted by 5 min breaks inhaling air from an oronasal mask. Then they were decompressed for 7-10 min to normal ambient pressure. FE_{NO} was measured at flow rates from 30 to 250 $mL \cdot s^{-1}$ ~30 min before and ~15 min after HBO treatment followed by FVC and FEV_1 (Vitalograph Ltd., Buckingham, England). All patients had breakfast at 7 am and the treatment took place between 9 and 11 am.

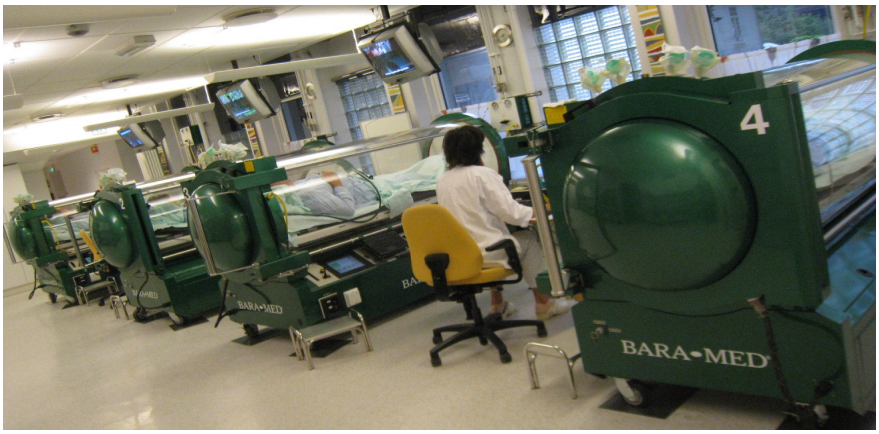

Figure 3: *Monoplace hyperbaric chambers at Haukeland University Hospital, Bergen, Norway.*

Twenty healthy subjects recruited from UIB were exposed to NBO. They sat passively with a nose clip breathing 100% oxygen at normal atmospheric pressure (PO_2 = 100 kPa) through two-way non-rebreathing T-shape™ valve (Hans Rudolph, inc. Kansas City, USA). The oxygen was provided from a compressed source via a demand system connected to the T-valve. The gas was humidified with water thru a container before it reached a reservoir (figure 4). The reservoir was supervised and the gas flow was adjusted for each subject. In the control exposure the subjects were breathing air directly from the atmosphere. FE_{NO} at flow rates from 30-250 $mL \cdot s^{-1}$ was measured ~15 min before and ~15 min after exposure followed by FVC and FEV_1 (Vitalograph Ltd., Buckingham, England). FE_{NO} at 50 $mL \cdot s^{-1}$ was also measured during exposure at 30, 60 and 90 min. In addition, a group of six healthy subjects recruited from NSSS performed a control

study breathing ambient air for 90 min. The exposure was performed 1 hour after last meal between 3 and 6 pm.

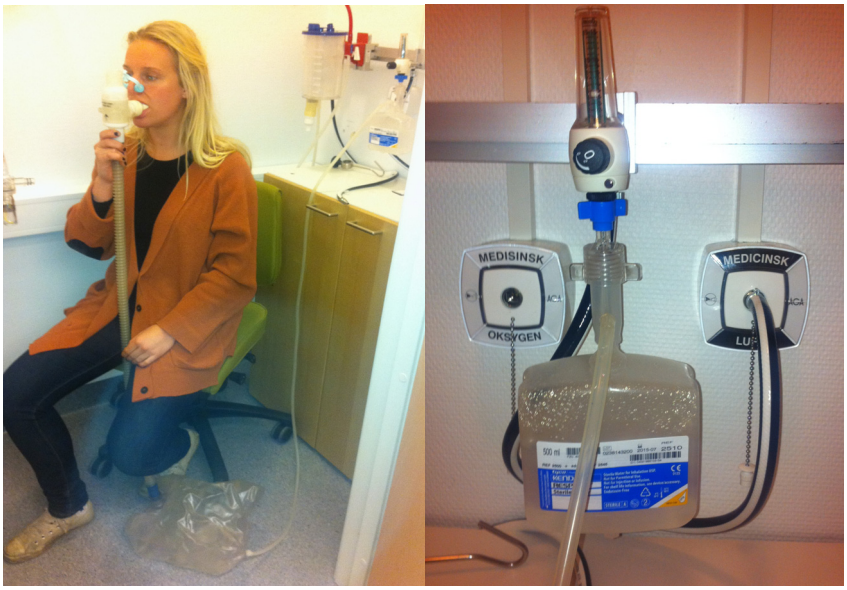

Figure 4: *The breathing system during exposure to normobaric hyperoxia at Haukeland University Hospital, Bergen, Norway.*

Paper III

The data was collected at the respiratory laboratory at HUH in November 2010. Eighteen healthy subjects were recruited from UIB to perform an NBO exposure. The subjects sat passively for 90 min with a nose clip breathing 100% oxygen at normal atmospheric pressure (PO_2 = 100 kPa) as explained earlier in paper II. FE_{NO} and lung function was performed in the same sequence each time 30 min before and 15 min after exposure. The healthy subjects functioned as their own control breathing ambient air and were tested with the same protocol on a separate day. The exposure was performed 1 hour after last meal between 3 and 6 pm.

9.4 Measurements

Subjects were instructed to avoid exercise, drinking caffeine and intake of nitrate-rich food on the same day as the experiment. No food or liquid were allowed one hour before testing. During the test it was only allowed to drink water. Temperature and barometric pressure were collected before each test. All tests were performed under similar environmental conditions (20-23°C). There were maximum three weeks and minimum three days between each test. All testing equipment was calibrated daily and the measurements were performed according to the standards specified by the ATS/ERS (35,98,99).

9.4.1 Anthropometry

The subjects were dressed in light clothing and no shoes for height and body mass measurements. 1kg was drawn of due to the clothing. Height was measured to the nearest 0.5cm and body mass to the nearest 0.1kg. At NSSS stature and body mass were measured by a digital scale (Seca, Hamburg, Germany). At HUH stature was measured by stadiometer (Seca 216, Hamburg, Germany) and body mass by a weight system (Tanita Corparation, Tokyo, Japan).

9.4.2 Exhaled nitric oxide

Two different techniques were used to measure exhaled NO concentration. The first was a single-breath online technique that measured FE_{NO} with a chemiluminescence analyser (CLD 88sp, ECO MEDICS, Duernten, Switzerland). This method was performed during a single flow-controlled exhalation against a resistance. The subjects were instructed to stand, exhale before inhaling NO-free air via a mouthpiece to TLC, followed by an immediate exhalation against a counter pressure of approx.10 to12cm H_2O in 12 second into the NO analyser. For that period of time a plateau was reached at the end of the exhalation and the final 3 seconds were used to estimate the level of exhaled NO. The online analysis of the exhaled NO gas detects a plateau phase in accordance to the ATS/ERS guideline and stops the measurement. The time of the measurements depends on the patient size, typical 8 to 10 seconds for adults or 6 to 8 seconds for children.

The second method was an offline technique where exhaled NO was collected in an aluminized NO-impermeable reservoir Mylar® bag using a hand-held device (Nitric Oxide Offline Collection Kit, ECO MEDICS AG, Duernten, Switzerland). The same breathing maneuver as for online sampling was performed until the Mylar® bag was filled. In the first 5 seconds dead-space gas (250 mL) was discarded in a small reservoir and exhaled gas was collected in the Mylar® bag between 5 to 10 seconds (250 mL). A visible needle indicated the pressure level and helped to maintain the expiration at a constant flow rate. For further analysing, the Mylar® bag was disconnected from the hand-held device and connected by a sample line to the chemiluminescence analyser. All measurements were performed in triplicates and mean values of three successive measurements within 10% deviation were used in the analysis. During testing the level of NO in ambient air was held under observation. Confirmation of the calibration and flow rate of the sampling system was performed on a daily basis. There was a strong correlation between online and offline measurements of FE_{NO} pre and post exposure to altitude (figure 5).

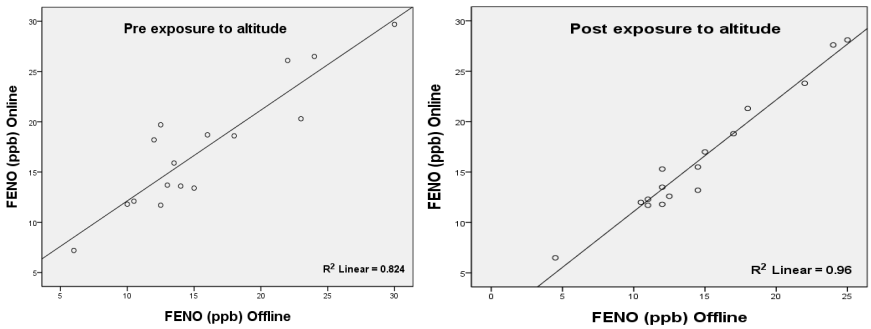

Figure 5: *The relationship between online and offline measurement of the fraction of nitric oxide in exhaled gas (FE_{NO}) pre and post exposure to altitude.*

PE_{NO} was corrected for increased expiratory flow rate at 2800m (PE_{NOcorr}) due to differences in gas density effects at altitude. The pressure drop over the resistor is proportional to gas density and the square of flow rate over the orifice in the resistor. The expiratory flow rate over the resistor was calculated to be 59 $mL \cdot s^{-1}$ at 2800m. FE_{NO} was converted to PE_{NO} (mPa) to express the molar concentration of NO ($PE_{NO} = FE_{NO} \cdot$ (pressure barometric – vapour pressure of water). There is a hyperbolic relationship between FE_{NO} and expiratory flow rate, and FE_{NO} is reduced with increasing flow rate (100). The next step in the correction procedure was then to estimate

what PE_{NO} measured at an expiratory flow rate of 59 mL·s^{-1} would be if measured at 50 mL·s^{-1}. Data from paper II where 20 healthy subjects measured FE_{NO} online at expiratory flows of 30-250 mL·s^{-1} were used to calculate the mean slope and intercept for the linear relationship between PE_{NO} and the inverse of expiratory flow rate. This relationship, $PE_{NO} = 0.51 + 63.3\ V'^{-1}$, where V' is flow rate, was used to correct PE_{NO} measured at altitude with an estimated expiratory flow rate of 59 mL·s^{-1} to 50 mL·s^{-1}, the correction factor being 1.12.

The mathematical model by Högman and Meriläinen was used to calculate C_ANO and $J_{aw}NO$ (34). Three single-breath tests at different expiratory flow rates were used for the calculations. FE_{NO} was measured online at flow rates of 30, 100 and 250 mL·s^{-1}. The three flow values, low, medium and high, and the corresponding FE_{NO} values were inserted into the algorithm provided.

9.4.3 Lung function

All lung function tests were performed in an indoor environment with regulated temperature and humidity by a trained technician. Volume and test gas calibrations were done before each test, and the calibration results were logged. The subject sat in an upright position using a nose clip to avoid any leakage. All measurements were done in the same order each time beginning with FE_{NO}, and then the single-breath oxygen test, D_LCO, the multi-breath nitrogen washout test ending with dynamic lung volumes. All lung function variables were calculated with the algorithms provided with the software and the measurements followed the criteria of the ERS (101).

Distribution of ventilation

The single-breath oxygen test estimates the uneven distribution of ventilation in the lung by measuring closing volume (CV) after a single inhalation of 100% oxygen from residual volume (RV) to TLC, and the slope of phase III of the nitrogen washout curve calculated over the mid-expiratory range (figure 6). The slope of phase III is generally taken when VC has been expired by 30% to the beginning of phase IV. The single-breath oxygen test begins with stable tidal

breathing expiration to RV and then a full inspiration of 100% oxygen ending with a self-monitored slow exhalation of 50 $mL \cdot s^{-1}$ to RV. CV and the slope of phase III of the nitrogen washout curve may detect small airways dysfunction.

Diffusion capacity for carbon monoxide

The intra-breath method was used to measure D_LCO, which is the transfer of carbon monoxide from the alveolar gas to the haemoglobin in the lung capillaries (102). The test starts with normal breathing before the subject slowly expires to RV followed by a rapid inhalation to TLC. During this manoeuvre the subject inhaled a known gas mixture that contains 0.3% CO, 0.3% methane, 0.3% acetylene and 21% oxygen in nitrogen. Further, the subject expires slowly, without any breath-hold, at a target flow rate of 500 $mL \cdot s^{-1}$ to RV following an on-screen flow indicator. The test gas concentrations were measured during exhalation. Effective alveolar volume was measured from the single-breath dilution and washout of methane, and D_LCO and pulmonary blood flow based on the rate of change of CO and acetylene concentration (103). Effective alveolar volume is referred to as the total volume of gas accessible for exchange with blood under normal conditions (104). The diffusion coefficient for carbon monoxide was calculated as a $D_LCO \cdot V_A^{-1}$.

Static lung volumes

The multi-breath nitrogen washout test was used to measure static lung volumes. In addition, give information related to distribution of ventilation that was derived from the nitrogen washout curve. The test measures functional residual capacity (FRC). The subject starts with normal tidal breathing. When a stable end-expiratory volume is reached the apparatus switched to breathing 100% oxygen until all the nitrogen was washed out of the lung (103). Next the subject performed a full inspiration to TLC followed by a full expiration to RV. The difference between FRC and expiratory reserve volume was calculated as RV, and TLC as the sum of inspiratory capacity and FRC. VC is measured during a slow expiration and is the maximal air a subject can expire from the lungs after a full inspiration (99).

The nitrogen washout time is the time used to bring end tidal nitrogen concentration below 2%, and the lung clearance index was the number of turnovers of FRC to bring it below 2%. In healthy young subjects the lung clearance index is in range of 5-9, whereas it increases in older subjects (104). The nitrogen washout time and lung clearance index were regarded as indices of distribution of ventilation.

Dynamic lung volumes and maximal expiratory flow rates

Spirometry was measured by maximal forced expiratory flow volume curves and used to measure dynamic lung volumes and maximal expiratory flow rates. The highest values taken from at least three satisfactory forced expiratory manoeuvres from TLC were used to measure FVC, FEV_1 and peak expiratory flow rate. The highest values from flow-volume loops was used to record maximal expiratory flow rates at 25, 50 and 75% of FVC expired and mean mid-expiratory flow rate where FVC was not less than 95% of the highest FVC (99). Spirometry was done after the measurements of FE_{NO} (35). Predicted values for FVC and FEV_1 were estimated by local reference values (105).

9.4.4 Relativ humidity and filter test

Nitric oxide is soluble with water, which may influence FE_{NO} levels. The water vapour in the Mylar® bags was measured to assess if temperature fluctuation with recompression from altitude could have led to condensation of water vapour inside the bag. The hand-held device from ECO MEDICS AG (Duernten, Switzerland) was used to sample exhaled gas at sea level with two different filters (Air™ Vickers Industrial Estate, Lancashire, UK; Smiths Medical International Ltd., Kent, UK). To prevent humidity the bag was flushed with compressed gas, which was bone dry. The humidity sensor (FLUKE®, 77 Multimeter, ELFA Distrelec, Sweden) was calibrated and connected to a power supply (Danica Supply AS, TPS23a, Denmark). The bag was immediately analysed with humidity and temperature probe (HMP50, Vaisala Intercap®, Helsinki, Finland) directly connected to the bag. Relative humidity was 80-95% indicating that samples taken at sea level and at altitude were equally influenced by water content in the sampling bag.

FE_{NO} was measured with three different filters in six healthy subjects (Air™, Vickers Industrial Estate, Lancashire, UK (17.4 ± 5.4 ppb), Sterivent, Tyco Healthcare, MO, Italy (17.4 ± 5.5 ppb) and HME, Smiths Medical International Ltd., Kent, UK (21.4 ± 8.0 ppb)). Repeated measures ANOVA showed no difference between the three filters (p = 0.21).

9.5 Statistics

The particular statistical procedures used are given in the respective papers. Statistical analyses were performed in Statistical Package for Social Sciences Version 18 (SPSS, Chicago, IL, USA). Data was expressed as mean ± standard deviation and a *P* value of < 0.05 was considered significant. Paired-samples t-test was used for comparison of FE_{NO} before and after exposures to hyperbaric and normobaric hyperoxia, hypobaric hypoxia and when breathing ambient air. Repeated measures ANOVA for group differences was used for comparison of FE_{NO} and lung function variables obtained pre and post exposure to both NBO and when breathing ambient air, in addition to comparison of the different filters. In paper I the Bonferroni method was used to correct for multiple comparisons in exhaled NO and a p value < 0.01 was considered significant. Mixed models analysis (ANOVA) was used for repetitive measurements during the 90 min exposure to altitude and time of NO measurements as independent variables. This statistical model contains both fixed and random effects, and is useful to analyse the difference between and within subject's factors. Linear regression was used for comparison between online and offline measurements in FE_{NO} and estimation of the correction factor for PE_{NO} at altitude in paper I, in addition to FE_{NO} and lung function variables in paper III.

Sample size

The sample size was based on the difference of the change in FE_{NO} from previous studies (68,69). Taraldsøy et al. (69) found that a sample size of eight subjects was sufficient to detect a change in FE_{NO} by ~30% after exposure to HBO therapy with 80% power and a *P* value of < 0.05 was considered significant. A sample size of 16 subjects was calculated as necessary to detect a

sufficient change in FE_{NO} with 80% power and a significance level of 5% in all three papers. This was based on previous knowledge of the variation in FE_{NO} (± 4 ppb) assuming that the standard deviation of the difference between repeated measurements of FE_{NO} is 15% and a decrease in FE_{NO} of > 30% is thought as biologically relevant (68,69).

10. SUMMARY OF PAPERS

10.1 Paper I

Exhaled nitric oxide concentration upon acute exposure to moderate altitude.

Our main objective was to assess the immediate changes in the partial pressure of nitric oxide in exhaled gas in healthy trained subjects who were exposed to moderate altitude. We hypothesized that PE_{NO} was reduced during exposure to moderate altitude at rest.

One group of 20 and a smaller group of 9 healthy subjects were exposed to an ambient pressure of 728 hPa (546 mmHg) corresponding to an altitude of 2800m for 90 and 5 min, respectively, in an altitude chamber. FE_{NO} was measured online before and after exposure to altitude. PE_{NO} was measured offline before, during exposure at 5, 30, 60, 90 min and after exposure by sampling exhaled gas in tight metal foil bags. A correction for increased expiratory flow rate due to gas density effects at altitude was performed. The healthy subjects functioned as their own control breathing ambient air and were tested at sea level with the same protocol on a different day.

PE_{NO} was significantly decreased by 13-16% ($p = 0.002$) and FE_{NO} increased by 16-19% at altitude ($p = 0.004$). However, there was no significant change in PE_{NOcorr} after exposure to altitude for 5 min ($p = 0.16$) and 30 min ($p = 0.12$) compared to baseline at sea level. FE_{NO} was significantly increased ($p < 0.001$) during exposure to altitude compared to baseline values, but no change in the control breathing ambient air. Mixed model analysis confirmed unchanged PE_{NOcorr} ($p = 0.77$) and the reduction in PE_{NO} ($p = 0.002$).

There was no significant change in PE_{NO} after correcting for gas density effects on expiratory flow rate during exposure to an altitude of 2800m.

10.2 Paper II

Bronchial nitric oxide flux and alveolar nitric oxide concentration after exposure to hyperoxia.

The purpose of this study was to partition FE_{NO} into its flow-independent alveolar and bronchial components in patients exposed to HBO therapy and in healthy subjects exposed to NBO breathing 100% oxygen.

12 patients undergoing HBO therapy at a PO_2 of 240 kPa and 20 healthy subjects exposed to NBO breathing 100% oxygen were compared to a control group of 6 subjects breathing ambient air, all for 90 min. FE_{NO} was measured at flow rates from 30 to 250 $mL \cdot s^{-1}$ before and after the exposures. The mathematical model developed by Högman and Märilainen was used to calculate $J_{aw}NO$ and C_ANO.

After a single HBO treatment session FE_{NO} at 50 $mL \cdot s^{-1}$ was reduced by 30 ± 9% ($p < 0.001$) and by 25 ± 9% after breathing 100% oxygen ($p < 0.001$). There was a gradual decrease at 60 and 90 min approaching the 15 min post-exposure value. There was a significant reduction in $J_{aw}NO$ ($p < 0.001$), but no change in C_ANO after exposure to HBO therapy ($p = 0.47$) or NBO ($p = 0.44$).

The reduction in FE_{NO} after exposure to normobaric and hyperbaric hyperoxia appears to be predominantly an airway effect. An unchanged and low C_ANO indicate preserved integrity of the gas exchange units without increased alveolar dead space at rest.

10.3 Paper III

Exhaled nitric oxide and lung function after moderate normobaric hyperoxic exposure.

Pulmonary oxygen toxicity is associated with inflammatory responses in the airways and alveoli. The purpose of this study was to investigate if the changes in FE_{NO} after exposure to NBO breathing 100% O_2 at 1 ATA for 90 min, are associated with changes in lung function.

Eighteen healthy non-smoking subjects were exposed to NBO breathing 100% oxygen and breathing ambient air both for 90 min on separate days and in random order. The lung function

measurements were performed in the same sequence each time before and after the exposures; FE_{NO}, the single-breath oxygen test, D_LCO, the multiple-breath nitrogen washout test and, finally, dynamic lung volumes.

There was no association between the decrease in FE_{NO} by 20% ($p < 0.001$) and the change in lung function variables after exposure to 100% oxygen at normal atmospheric pressure for 90 min.

10.4 Summary of the results in nitric oxide in exhaled gas

Table 5 includes the results from the three separated papers in this thesis. FE_{NO} was measured before and after exposure to hypobaric hypoxia (PO_2 = 15 kPa), normobaric hyperoxia (PO_2 = 100 kPa), hyperbaric hyperoxia (PO_2 = 240 kPa) and when breathing ambient air (PO_2 = 21 kPa), all for 90 min.

Table 5: *The fraction of exhaled nitric oxide (FE_{NO}) before and after exposure to hypobaric hypoxia for 5 (HH_5) and 90 min (HH_{90}), normobaric hyperoxia (NBO), hyperbaric hyperoxia (HBO) and control study breathing ambient air, all for 90 min (AA).*

Exposure	FE_{NO} (ppb) pre	FE_{NO} (ppb) post	Δ FE_{NO}	p-value
HH_5 (15 kPa) [1]	18.6 ± 8.2	18.8 ± 8.4	↑1 ± 22%	0.82
HH_{90} (15 kPa) [1]	15.8 ± 5.8	15.2 ± 5.3	↓3 ± 15%	0.33
NBO (100 kPa) [2]	17.8 ± 6.2	13.3 ± 5.2	↓25 ± 9%	< 0.001
NBO (100 kPa) [3]	19.5 ± 7.4	15.2 ± 5.8	↓20 ± 20%	< 0.001
HBO (240 kPa) [2]	17.6 ± 8.3	12.3 ± 6.3	↓30 ± 9%	< 0.001
AA (21 kPa) [1]	15.7 ± 6.3	16.0 ± 5.9	↑6 ± 18%	0.58
AA (21 kPa) [3]	21.6 ± 8.8	20.1 ± 8.2	↑5 ± 14%	0.04

The numbers in superscript indicate the paper the results are based on.

Figure 6 shows the dose-response relationship between oxygen exposure and the change in FE_{NO}. The figure includes the results in FE_{NO} after exposure to hypobaric hypoxia, normobaric hyperoxia and when breathing ambient air in the healthy subjects, all for 90 min. In addition, the results from the study by Fothergill & Gertner (70), where subjects were exposed to a PO_2 of 203 kPa for 6 and 8 hours in a hyperbaric chamber, where included. Figure 6 is an exponential curve and the regression equation for FE_{NO} % change = $95.786 * e^{-0.001 * \text{oxygen dose}}$. The curve estimation has a good fit ($r^2 = 0.95$) and the coefficient for oxygen dose was highly significant ($p < 0.001$).

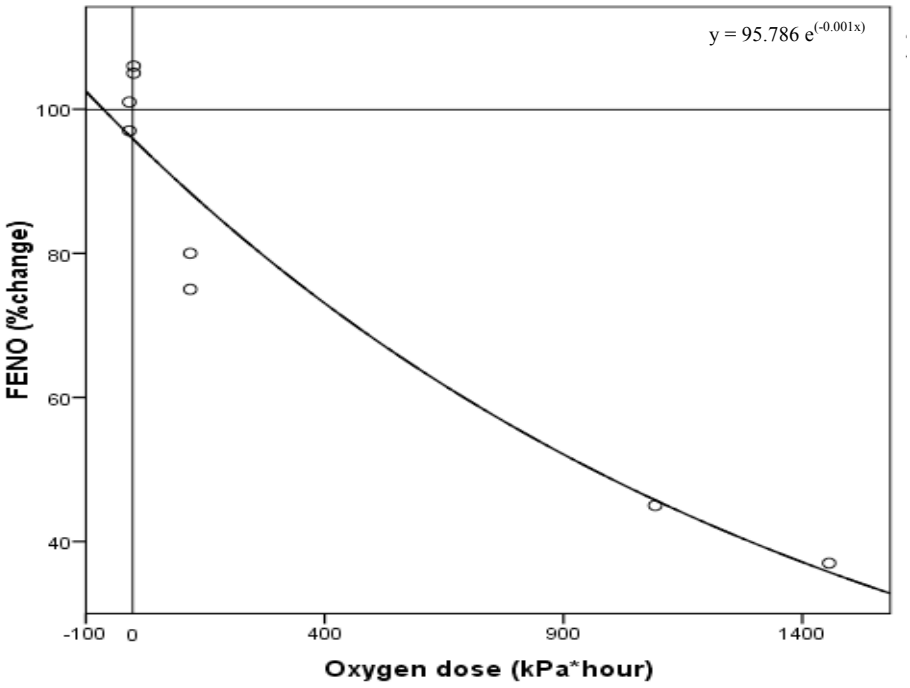

Figure 6: *The dose-response relationship between oxygen exposure and the change in the fraction of nitric oxide in exhaled gas (FE_{NO}).*

11. DISCUSSION

The first section integrates and discusses the results of the three papers and the main objective of the present thesis, whereas the second part discusses the methodological issues.

11.1 Discussion of the results

1. The dose-response relationship between oxygen exposure and the change in FE_{NO} during and after hypoxia and hyperoxia in healthy humans.

In this thesis there was a gradual decrease in FE_{NO} during a 90 min exposure to normobaric hyperoxia. Throughout the same time course when exposed to hypobaric hypoxia no change was found after correcting for gas density effects and the change in expiratory flow rates.

However, there was an immediate increase in FE_{NO} at arrival at 2800m for 5 min, which continued throughout the exposure. Vinnikov et al. (62) reported no change in FE_{NO} measured at baseline in Bishkek at 780m and the first day at altitude. A recent study by MacInnis et al. (71) found no change in FE_{NO} during the first hour of exposure to normobaric hyperoxia. However, FE_{NO} was significantly increased from 2-6 hours and steady throughout the observation period. They indicated that biochemical and inflammatory mechanisms related to hypoxic exposure and mountain sickness probably has a longer time course than the immediate physical effects of exposure. This may indicate a dose-dependent response in FE_{NO}, but the time course of changes in FE_{NO} needs further investigation. The difference in our results in paper I and the results reported by Vinnikov et al. (62) and MacInnis et al. (71) might be due to the difference in experimental protocols used.

PE_{NO} was significantly decreased by 13-16% during exposure to 2800m of altitude. However, after correcting for altitude conditions PE_{NOcorr} showed only a non-significant reduction of 4-6% after 5 and 30 min of exposure. This was similar to or somewhat smaller than previous studies that found a trend towards a reduction in PE_{NO} of about 10% at altitudes of 1500-3100m (61,64). Hemmingsson & Linnarsson (64) reported no change in PE_{NOcorr} at 1500m, but they found a decrease in FE_{NO} by 15-33% during exposure to 3100-5000m in a hypobaric pressure chamber when compared to normobaric hypoxia. This was similar to a study by Brown et al. (61) who

reported a reduction in PE_{NOcorr} by 10-21% at 2800-4200m compared to sea level. That was a field study where expiratory flow rate was controlled and the subjects were transported by car to 2800m and 4200m. There was no further change in PE_{NOcorr} during the 3 hours stay at 4200m, and PE_{NOcorr} returned to baseline immediately upon arrival at sea level. Recently, two studies reported a gradual reduction in PE_{NO} over an extended time course at altitude (62,63). Vinnikov et al. (62) found a larger reduction in PE_{NOcorr} measured at 3800m of altitude the 21st day compared to the 3rd day. Donnelly et al. (63) showed a progressive decrease in PE_{NO} during a graded ascent to altitude up to 5050m. This may suggest a dose-response relationship with larger reductions in PE_{NO} at higher altitudes, and the partial pressure of oxygen itself may influence the results in a smaller way than previously reported.

It has been speculated that decreased availability of oxygen during hypobaric hypoxia may reduce the production of NO. The studies of normobaric hypoxia with partial pressures of oxygen corresponding to altitudes up to 5000m and normal gas density do not support a hypoxic mechanism for the reduced PE_{NO}. Hemmingsson and Linnarsson (64) demonstrated that the reduction in PE_{NO} was related to the increasing diffusivity of NO with decreasing gas density supporting a physical effect with increased axial backdiffusion of NO into the alveoli. However, there may be inter-individual differences in that the time course of the changes in PE_{NO} during the stay at altitude appears to be different in subjects susceptible to high altitude pulmonary edema compared with those not susceptible (106). High altitude pulmonary edema susceptible subjects had a gradual decrease in pulmonary NO excretion during exposure to hypoxia with a fraction of oxygen in inspired gas of 0.12 for 2 hours, whereas non-susceptible subjects had no change.

In this thesis $FE_{NO}$was significantly reduced by 30% after hyperbaric hyperoxia and by 20-25% after normobaric hyperoxia, whereas no change was found in FE_{NO} after exposure to hypobaric hypoxia and when breathing ambient air, all for 90 min. There are some conflicting results regarding normobaric hyperoxia, where a decrease (65,66), an increase (67,72) and no change have been found (65). The reduction in FE_{NO} by 30% after exposure to hyperbaric hyperoxia is similar to the majority of studies that reported a reduction between ~30-70% (65,69,70). Fothergill & Gertner (70) reported a decrease by 55-63% in FE_{NO} after exposure to a PO_2 of 203 kPa for 6-8 hours in eight subjects (70). The results from the healthy subjects in this thesis together with the results from the study by Fothergill & Gertner (70) are included in figure 6. The

patients undergoing HBO therapy were not included because of their smoking history, age difference, heterogeneity of the group and possibly interference of radiation therapy. In these patients FE_{NO} was reduced by 30% after 90 min at 240 kPa, which was similar to the predicted curve estimation in the dose-response model. The results may indicate a dose-dependent relationship between PO_2 and the change in FE_{NO}, where FE_{NO} is decreasing with increasing oxygen dose.

2. The alveolar and bronchial contributions to the changes in FE_{NO} after exposure to normobaric and hyperbaric hyperoxia in humans.

After exposure to hyperoxia at PO_2 of 100 and 240 kPa for 90 min FE_{NO} was reduced by 25% and 30%, respectively. There was no change in C_ANO, but a significant reduction in $J_{aw}NO$ after exposure to hyperoxia. This was in agreement with a previous study by Fothergill & Gartner (70). They demonstrated the same result in healthy divers exposed to a PO_2 of 203 kPa for 6 hours in a hyperbaric chamber. The reduction in FE_{NO} after exposure to hyperoxia appears to be predominantly an airway effect. An unchanged and low C_ANO indicate preserved integrity of the gas exchange units without increased alveolar dead space at rest. Reduced small airways conduction is an early sign of development of pulmonary oxygen toxicity, which could be consistent with reduced FE_{NO} in the bronchial compartment.

Some inflammatory processes in the lung are associated with an increased production of NO. As stated earlier, pulmonary oxygen toxicity is associated with inflammatory responses in the airways and in the alveoli. We can not conclude whether the reduction in FE_{NO} is associated with structural changes in the lung or not. However, there might be biochemical changes. NO may either be chemically scavenged in a hyperoxic environment or its synthesis may be inhibited. Tetrahydrobiopterin is required in the NO synthesis, and when oxidised to dihydrobiopterin it will no longer functional as cofactor (figure 1). Further, it may be speculated that exposure to high partial pressure of oxygen results in reduced NO production in all three isoforms of NOS influencing both vascular and neurogenic activity.

3. The relationship between the change in FE_{NO} and the changes in lung function after exposure to normobaric hyperoxia in healthy humans.

Despite a significant reduction in FE_{NO} by 20% no change was found in indices of distribution of ventilation, maximal expiratory flow rate or D_LCO. This may suggested no small airways' dysfunction and preserved gas transfer in the lung. This was supported by the findings in paper II, where no change in C_ANO was found and the reduction in FE_{NO} appears to be predominantly an airway effect. Previous studies reported no correlation between FE_{NO} and lung function test in patients with asthma (107,108). Though, Sippel et al. (109) found a weak correlation between FE_{NO} and FEV_1.

A dose-response relationship is established between oxygen exposure measured as UPTD and the reduction in VC (110). Nevertheless, a decrease in D_LCO and maximal expiratory flow rates has been found after exposure to hyperoxia where changes in VC were neither found nor predicted. The data in paper III was not sufficient to determine or predict pulmonary oxygen toxicity, and a change in VC was not expected with the oxygen exposures used. However, changes in other lung function variables need more research.

Is FE_{NO} a useful marker to predict pulmonary oxygen toxicity?

FE_{NO} was investigated to study the possibility to function as a new marker to predict pulmonary oxygen toxicity. This was not confirmed with the oxygen exposures used. The results from the present thesis showed that the reduction in FE_{NO} by 20% was not associated with changes in lung function variables, but the hyperoxic exposure was low and no change in VC was predicted. Therefore, FE_{NO} might be its own index of oxygen exposure rather than a direct marker of pulmonary oxygen toxicity. Further studies over a wide range of oxygen exposures are necessary to establish the role of FE_{NO} as a marker of pulmonary oxygen toxicity.

11.2 Methodological considerations

11.2.1 Study design

This thesis includes the results from a randomized controlled trail (paper I and III) and a non-randomized trial in subjects from Norway (paper II). The crossover design is an appropriate method to detect differences in FE_{NO} and lung function after exposure to different oxygen exposures. In paper I and III, the subjects functioned as their own control and the exposure was drawn in random order, which reduced the random variance.

The healthy subjects included from UIB and NSSS were similar relative to anthropometrical measurements, age and physical activity. However, the patients included from HUH was significantly older compared to the healthy subjects in paper II. The healthy subjects were not exposed to HBO due to ethical considerations. The risk of side effects i.e. decompression illness was considered unacceptable. In previous studies patients having HBO therapy have been compared with matched control groups with respect to age, gender and smoking habits (68,69).

11.2.2 Reliability

In statistics, reliability is important when it comes to describe the consistency or the repeatability of the data collection. The reliability of a measurement or a test defines its capacity to create the same results repeatedly under the same circumstances. The variation in measurements when taken by different personnel, but with the same method or equipment is called inter-rater reliability.

The most used method in this thesis was the online measurement of $FE_{NO.}$ Kharitonov et al. (111) showed that the reproducibility in FE_{NO} was high for repeated measurements and the intra-subject reproducibility was good. There are disagreements about diurnal variation of exhaled NO. Kharitonov et al. reported no day to day or diurnal variation of exhaled NO in healthy and asthmatic humans (111). On the contrary, a small increase consistent with the demonstration of a diurnal variation with an increase in FE_{NO} of ~15% from the morning into the afternoon has been reported (112,113). The subjects performed the tests at the same time of the day in each study to avoid any diurnal changes in FE_{NO}. In paper 1 and 3 diurnal variability in FE_{NO} was estimated

from the online measurements before and after the control study breathing ambient air. Day-to-day variability in FE_{NO} measured as the difference between baseline measurements on the two days collected before exposure at 9 am in paper 1 and at 3 pm in paper 3. No diurnal or day to day variation was found and the change in FE_{NO} was less than 10% and < 2.1 ppb in both papers. This is within normal variation in FE_{NO} and within clinically satisfactory limits (± 4 ppb) (114).

In this thesis day to day variation in lung function variables was < 7% and the diurnal variation was < 5%. There was a small significant reduction in FVC of 0.06 and 0.05L after oxygen exposure in paper I and II, respectively. This was probably normal within subject variability expected from repeated measurements performed on the same subject from day to day measurements. One technician was responsible for the data collection in this thesis. One additional technician performed some of the lung function tests in paper III. The reliability was not identified as a problem.

11.2.3 Validity

Reliability does not indicate validity. A measurement with high validity means to which degree a test is actually measuring what it is expected to measure (115). Validity is normally separated into external and internal validity. The external validity of a study refers to the generalizability, which concerns the ability to draw valid conclusions about a large population based on the results of the study (116).

The subjects were mainly recruited by oral presentations through lectures and information spread by word of mouth. Whether the recruitment strategy successfully included a representative sample is not known. Nevertheless, personal contact showed to include more participants than written material (invitation by e-mail). The students recruited in this thesis are probably not representative for the general Norwegian population. They might be in better physical condition and leaner compared to the average Norwegian in the same age group (117). Therefore, the results should be generalized with care.

Internal validity of a study refers to the ability to draw valid conclusions concerning the study population. It may be influenced by several factors such as systemic errors, random errors and confounding factors. In paper II, the patients had a larger variability in baseline FE_{NO} after exposure to HBO therapy. This could be due to the age differences and heterogeneity of the group.

The information about disease, food intake, earlier smoking habits and exercise was self-reported and may reduce the validation of the study. Given the size of the study and previous studies with similar results, random errors and the subjects' self-report were not identified as a problem. However, systemic errors are independent of study size, and cannot be excluded.

11.2.4 Metodological strenghts

A major advantage in this thesis was that all data was collected mainly by one technician. FE_{NO} was immediately analysed and repetitive measurements without affecting NO formation was possible. To avoid possible influence of ambient NO with FE_{NO} values, NO-free air was inhaled (35). Nasal air contains high concentration of NO. Therefore, it is important to close of the velum during exhalation. The subjects were supervised during FE_{NO} measurements to prevent contamination of the sample with nasal air. During oxygen breathing a two-way non-rebreathing T-shape™ valve and a nose clip was used to prevent leakage. In all three papers the recommended expiratory flow rate of 50 $mL \cdot s^{-1}$ was used (35). Paper II also included expiratory flow rate between 30-250 $mL \cdot s^{-1}$.

The offline technique has shown to be comparable with online measurements (118,119). In this thesis there was a strong correlation between online and offline measurements of FE_{NO} measured before and after exposure to altitude (figure 5). Offline breath samples of FE_{NO} may easily be collected at any location and can be more practical than online measurements. It also provide similar sensitivity and specificity (120).

11.2.5 Methodological limitations

There are limitations in this thesis that need to be pointed out. The test protocol in paper I did not include measurements of exhaled NO immediately after arriving at altitude initially. Therefore, a smaller group was added later on to measure FE_{NO} when arriving at altitude to study the immediate effect in FE_{NO}. Only one group should have been subjected to all the measurements. In addition, a control group breathing 15% oxygen at sea level could have been added to strengthen the study.

Patients exposed to hyperbaric hyperoxia have an increased risk of respiratory infections due to the HBO treatment. Several patients had emerging infections and were excluded before the pre-test. Some of the patients were ex-smokers and could have reduced FE_{NO} levels associated to a lower total airway NO (52). Högman et al. (121) showed with a diffusion model that alveolar NO was increased in smokers, but NO flux from the airways was decreased. The mechanism that is affecting exhaled NO does appear to be reversible in smokers. The amount of HBO therapy sessions the patients had completed before they were tested was not equal. For the most part, the measurements were performed during the first week of the HBO treatment. Taraldsøy et al. (69) measured FE_{NO} before hyperbaric oxygen exposure on different days during the course of a 4 week HBO treatment period. They found no change in baseline FE_{NO}. in the study by Kjelkenes et al. (68), a complete recovery in FE_{NO} within 24 hours was confirmed The patient's amount of HBO sessions was not identified as a problem.

Changes in D_LCO may be seen immediately after and up to several days after exposure hyperbaric hyperoxia. Fothergill and Gertner (70) found a trend of a decrease in D_LCO the next day after exposure to PO_2 of 203 kPa for 6-8 hours, whereas FE_{NO} was returned back to normal. In paper III D_LCO was only measured 15 min after exposure. Extended measurements the day after could have given more information about the time course of changes in lung function variables as seen in the study by Caldwell et al. (92).

Female's menstrual cycle was not registered in the subjects participating in the three papers included in this thesis. Morris et al. (56) reported no temporal connection between menstrual cycle and NO production, which indicated that FE_{NO} was not modulated by estrogens. In a study

by Johannesson et al. (57) FEV_1 and FVC were significantly higher during menstrual cycle in 12 women with cystic fibrosis.

12. Conclusions

Based on the results presented in paper I - III the following conclusions can be drawn:

1. FE_{NO} was reduced by 20-30% after exposure to normobaric and hyperbaric hyperoxia. After correcting for gas density effects on expiratory flow rate, PE_{NO} was not changed upon arrival at moderate altitude in healthy subjects. There was no change in FE_{NO} after exposure to breathing ambient air. There seems to be a dose-dependent relationship between PO_2 and the change in FE_{NO}, where FE_{NO} is decreasing with increasing oxygen dose.

2. There was a significant reduction in bronchial NO flux, but no change in alveolar NO concentration after exposure to normobaric and hyperbaric hyperoxia for 90 min. The reduction in FE_{NO} after hyperoxia appears to be predominantly an airway effect.

3. There was no association between the reduction in FE_{NO} by 20% and the changes in lung function variables after exposure to normobaric hyperoxia for 90 min in healthy subjects.

The results in this thesis suggest that there is not sufficient evidence at the time being to confirm that FE_{NO} may function as a direct marker of pulmonary oxygen toxicity.

13. Future perspective

Based on the results in this thesis the following research topics should be investigated:

1. Further research is needed to better understand the potential relationship with FE_{NO} as a direct marker of pulmonary oxygen toxicity. Additional studies with different oxygen exposures and lung function variables measured over a wide range may provide this information.

2. Prospectively quantify the dose-response relationship between the change in FE_{NO} and oxygen exposures. It would be interesting to study the change of FE_{NO} after hypobaric and hyperbaric exposures in a lager scale to see if the curve estimation continues to be good.

3. Further studies are needed to investigate the change in FE_{NO} after saturation diving and the association with gas embolism. Gas embolism may appear during decompression from increased pressure where gas bubbles enter arteries or veins. Previous studies have reported that pulmonary gas embolism increased FE_{NO}. It would be interesting to study the effect on lung capillaries and reduced D_LCO, which further may influence the alveolar NO concentration.

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