# DETERMINATION OF ENDOGENOUS AND DIETARY-DERIVED NITRIC OXIDE PRODUCTION IN EXHALED AIR OF ADULT HUMANS

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**Abstract**

The production of nitric oxide (NO) has been demonstrated in the human body. Studies have elucidated that NO can either be produced endogenously via enzymatic action or through exogenous factors from dietary nitrate reduction. Exhaled nitric oxide (eNO) levels were also observed to be increased in inflammatory conditions, such as asthma and rhinitis, compared to normal physiological conditions. The aim of this study was to observe the production of NO in the body, the influence of dietary nitrate on eNO and factors affecting NO production. In this cross-sectional study, eNO in healthy, control participants (n=25) and compared to exhaled NO in participants with asthma (n=6) and participants with seasonal rhinitis (n=7). Exhaled NO in 20 healthy controls was also after supplementation with dietary nitrate. Asthmatic participant's eNO concentrations (41±25 ppb) were higher compared to eNO in the control participants (12±12 ppb; p=0.04). There was no difference between eNO concentrations in participants with rhinitis (30±31 ppb) compared to control participants (12±12 ppb; p=0.21). There was also no dose-response relationship between the amount of dietary nitrate (nitrate-rich beetroot juice) and eNO concentration where eNO concentrations of 31±16 ppb, 34±31 ppb and 38±30 ppb; p=0.42 were measured for 35 mL, 70 mL and 140 mL of beetroot juice, respectively. A transient increase in eNO was observed after 30 minutes post-nitrate ingestion (20±8 ppb) compared to baseline eNO concentrations (6±5 ppb; p=0.03). The use of antibacterial mouthwash also reduced eNO concentrations post nitrate-ingestion (18±15 ppb) compared to beetroot ingestion without prior use of mouthwash (34±31 ppb; p=0.02). Exhaled NO levels were not reduced after antacid administration (p=0.791). The results of this study may indicate that the transient impact of dietary nitrate on eNO could account for the variations demonstrated in literature.

Keywords: exhaled nitric oxide, asthma, rhinitis, beetroot juice, mouthwash, antacid

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**List of Abbreviations and/or Acronyms**

**eNOS:** endothelial nitric oxide synthase

**eNO:** exhaled nitric oxide

**iNOS:** inducible nitric oxide synthase

**nNOS:** neuronal nitric oxide synthase

**NO:** nitric oxide

**BRJ**: beetroot juice

**LRTI**: lower respiratory tract infection

**URTI**: upper respiratory tract infection

**MW**: mouthwash

**AA**: antacid

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**Dedication**

This study is dedicated to my parents, Francina and David Keendjele who constantly encouraged me and provided emotional and financial support.

To my two brothers, John and Nawa, my family, friends; and a special thank you to Nonjabulo Matomela who never failed to provide words of encouragement throughout this study.

Last, but not least, I dedicate this thesis to the Lord Almighty, without Him none of this would have been possible.

**Declarations**

I, Tuwilika PT Keendjele, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

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# Chapter 1

# 1 Introduction

## 1.1 Background of the study

Nitric oxide is a messenger molecule that is crucial in vasodilation of smooth muscles (1). It is produced in different cells around the body either by enzymatic or non-enzymatic pathways. Epithelial cells lining the respiratory tract were also shown to produce and temporarily store nitric oxide (2). Endogenous production of nitric oxide occurs via a classical pathway that requires nitric oxide synthase enzymes to catalyse the reaction between L-arginine and molecular oxygen that results in nitric oxide production (3). Studies have shown that inflammatory respiratory conditions such as asthma result in elevated levels of exhaled nitric oxide (4, 5). These observations in asthmatic individuals have instigated the use of exhaled nitric oxide levels as a marker of inflammation in the airways of asthmatic patients (6, 7). Alternatively, non-enzymatic production of nitric oxide was observed from the reduction of exogenous nitrates (i.e., from dietary sources) (8, 9). Dietary nitrates then enter a reducing pathway that leads to the formation of nitric oxide and other nitrogen oxides.

Nitric oxide was primarily found to be produced through the enzymatic action of nitric oxide synthase (NOS), which catalyses the reaction between L-arginine and oxygen (10, 11). Nitric oxide produced in the respiratory tract, especially by the enzymatic action of inducible nitric oxide synthase (iNOS), either diffuses through an aqueous layer into the lumen of the airways or diffuses in the opposite direction into the circulation surrounding the tissue (2). This diffusion into the airway lumen allows for the measurement of nitric oxide in exhaled air.

Moreover, the alternative pathway for nitric oxide production that is independent of enzymes was later discovered (12-14). In this pathway, ingested inorganic nitrate

($NO_3^-$) is reduced to nitrite ($NO_2^-$) and subsequently to nitric oxide (NO). Inorganic nitrates are mainly obtained from dietary sources (i.e., green leafy vegetables, beetroot and cured meats). Orally ingested nitrates were found to be reduced, by nitrate reductase bacteria found in the oral cavity, to nitrite (15). The nitrite that was produced is swallowed and reduced by the acidic pH of the stomach to nitric oxide and other nitrogen species (12). As a result of nitric oxide production from this pathway, many studies have focused on determining the potential benefits of dietary nitrate consumption, especially regarding physical activity and cardiovascular conditions due to the vasodilatory role of nitric oxide in the body (13, 16).

With guidelines and recommendations from the European Respiratory Society (ERS) and the American Thoracic Society (ATS) (17), many studies have measured exhaled nitric oxide using a single breath manoeuvre where participants exhale at a certain flow rate, as it was found to affect the accuracy of exhaled nitric oxide measurements (18-22). Nitric oxide from the trachea or from the alveoli can be distinguished depending on the flow rate used during the exhalation manoeuvre (23-25).

Exhaled nitric oxide (eNO) is commonly measured in certain populations as a non-invasive method for detecting inflammatory conditions in the lungs such as asthma. Clinical measurements of eNO are mainly done in developed countries, whereas developing countries barely measure eNO clinically, due to the scarcity and cost of the equipment. In actual fact, in order to determine clinical significance of eNO, this same parameter should be measured in healthy participants. That being so, there is an abundance of eNO data collected from healthy individuals in populations in developed countries. Consequently, due to the paucity of eNO data from healthy individuals in developing countries, clinicians may be less likely to measure eNO in local patients.

An alternative source of nitric oxide is found in dietary nitrates. Studies have thus considered whether nitrate-rich foods have a physiological effect, specifically by observing blood pressure as well as nitric oxide concentrations in the blood and in exhaled air (26, 27). Furthermore, the non-enzymatic production of nitric oxide from ingested inorganic nitrates can be observed by measuring nitric oxide concentrations in exhaled air of humans (28). The non-enzymatic pathway can be confirmed by modulating different aspects that contribute to the production of nitric oxide, that is by altering the pH of the stomach or eliminating the bacterial action in the oral cavity (29, 30). The aim of this study is therefore to observe the kinetics of nitric oxide from exhaled air through the non-enzymatic pathway, by modulating physiological aspects (i.e., oral bacterial flora) within the pathway.

### 1.2 Statement of the problem

With the crucial role that nitric oxide (NO) institutes in vasodilation of smooth muscle, researchers focused on the potential benefits of dietary nitrates on physiological parameters (i.e., exercise and cardiovascular health) that improved because of vasodilation and increased blood flow (16, 31). The exact effects of dietary nitrates in the human body have been variable and inconclusive amongst studies, where some studies reported benefits such as improved exercise performance or through reducing blood pressure both in normal volunteers and patients with hypertension (16, 26, 31-36) while others reported no change (37-39). In most studies (16, 26, 31-39), dietary nitrate effects were investigated by measuring plasma nitrate and nitrite with the intention of correlating them to the physiological parameter being studied. There is a lack of research that sought to measure exhaled NO after ingestion of dietary nitrate, especially in healthy individuals, as most studies that measured exhaled NO levels were conducted on individuals with inflammatory respiratory conditions (4-7, 40). The

purpose of this study was therefore to examine the effects that dietary nitrate has on exhaled NO production in a healthy adult population from the University of Namibia, Hage Geingob Campus.

### 1.3 Objectives of the study

The primary objective was to observe the pathway of non-enzymatic production of nitric oxide by measuring its concentrations in exhaled air, post-ingestion of inorganic nitrates in the form of beetroot juice.

The specific objectives were as follows:

1. To differentiate between the nitric oxide sourced from the two compartments (i.e., bronchial and alveolar) of the respiratory tract.
2. To demonstrate the technique of measuring exhaled nitric oxide using chemiluminescence and electrochemical detection in healthy participants, asthmatic and participants with rhinitis.
3. To determine the effect of beetroot juice ingestion on exhaled nitric oxide.
4. To determine the effect of oral bacteria and stomach pH on nitric oxide production after ingestion of beetroot juice.

### 1.4 Hypotheses of the study

The study aimed to determine all of the hypotheses that are stated below.

1. Exhaled nitric oxide measured from the bronchial airways would be higher than that measured from the alveolar airway.
2. After ingestion of inorganic nitrate, in the form of beetroot juice, exhaled nitric oxide would increase.
3. The reduction of oral bacteria would decrease exhaled nitric oxide concentrations.
4. Increasing stomach pH would decrease exhaled nitric oxide concentrations.

## 1.5 Significance of the study

This study demonstrated the technique of measuring exhaled nitric oxide in both the laboratory and at home. A comparison between healthy participants and participants with inflammatory respiratory conditions (asthma and rhinitis) was made, which may be used to compare with published eNO values from other populations. The observations made in this study can be used as a support of the concept not only for the technique, but for its potential use by clinicians as a diagnostic tool for inflammatory airway conditions, such as asthma, as well as a home-based monitoring tool for people with inflammatory respiratory conditions. Moreover, this study aided in filling the gap of knowledge around the effects of dietary nitrates on exhaled nitric oxide.

## 1.6 Limitations of the study

This study was limited to a small sample size due the selection of homogenous sample groups. Due to the COVID-19 pandemic only a limited number of participants were recruited for the study. A larger study may be conducted in future to measure exhaled nitric oxide in the general population. Another limitation was that despite informing participants about the dietary restrictions, they may not have adhered to those restrictions.

## 1.7 Delimitations of the study

The study focused on three sample groups; healthy participants, participants diagnosed with asthma and participants with rhinitis. The healthy participants were non-smokers, had not experienced any infections (i.e., respiratory or gastric) for at least two weeks prior to the research experiments and were not on any medication. Respiratory infections generally have an effect on exhaled nitric oxide, however this study was

limited to asthmatics and individuals with rhinitis as they have different affected production sites of nitric oxide in the airways.

# Chapter 2

# 2 Literature Review

## 2.1 History of nitric oxide discovery

Nitric oxide gas was discovered over 200 years ago by Joseph Priestly (41) and was known as a toxic gas in the atmosphere. In 1980, Furchgott and Zawadzki (42) were studying the effects of acetylcholine on smooth muscle and found that a factor in the epithelium contributed to vasodilation and referred to it as endothelial-derived factor (EDRF). Concurrently, Katsuki and Murad (43) determined several agents that activated guanylate cyclase, including sodium nitrite, sodium nitroprusside and nitroglycerin, which in turn increased cyclic guanosine 3,5-monophosphate levels resulting in the relaxation of bovine tracheal smooth muscle. This work was followed up by Ignarro et al. (44) with the hypothesis that the relaxation of vascular smooth muscle caused by nitroprusside was mediated by nitric oxide, which they associated with the activation of guanylate cyclase. Both Ignarro and Moncada's research teams then independently identified EDRF as nitric oxide by comparing the chemical properties and actions of both molecules (3, 44). Palmer et al. (45) also demonstrated that nitric oxide was produced from L-arginine in porcine endothelial cells, a discovery that paved the way for the identification of nitric oxide synthase (NOS) enzymes that catalyse the production of nitric oxide from L-arginine.

In more recent years Lundberg et al. (29) demonstrated that NO could be produced independently from NOS via inorganic nitrate and nitrite in the human body. The use of inorganic nitrates in the body was first demonstrated in ancient Chinese medicine for the treatment of angina over 3000 years ago (46). Approximately two centuries later, Haldane (47) demonstrated that the red colour in meat was due to the binding of nitric oxide to haemoglobin, forming nitroso-haemoglobin. He further described that

nitrite converts deoxygenated haemoglobin to nitroso-haemoglobin, which he also observed to occur in salted meat (47). However, until recently it was believed that nitrate and nitrite were inactive metabolites of nitric oxide until researchers discovered the reduction of nitrate and nitrite to NO in vivo (48).

Studies also demonstrated nitrates and nitrites played a crucial role in cardiovascular health (31, 33, 49). An early study in rats, measured nitric oxide production after the induction of ischaemia in rat hearts (48). Zweier et al. (48) detected a weak nitric oxide signal in the rat hearts that were perfused normally compared to the ischaemic rat hearts, which produced a greater nitric oxide signal. This demonstration of nitric oxide formation during ischaemic conditions emphasised the non-enzymatic production of nitric oxide (48). It was then realised that these metabolites that were initially thought to be inactive, serve as storage molecules of nitric oxide (50). With the understanding of the non-enzymatic production of nitric oxide, nitrite specifically was demonstrated to be a major storage component of nitric oxide (50, 51). Dejam et al. (50) also noted the difficulty in directly measuring nitric oxide due its short half-life as a consequence of its reaction with haemoglobin that forms nitrate and methaemoglobin. Another study (52) measured nitrite and other nitrogen species in circulation after blocking nitric oxide production, from the NOS pathway. Gladwin et al. (52) found a reduction of blood flow in the forearm upon inhibition of NOS. They also found arterial-venous gradients for circulating nitrite, which indicated the bioactivity of nitrite as it also increased with the inhibition of enzymatic nitric oxide production (52).

Research then focused on the effects of inorganic nitrate on the cardiovascular system (31, 33, 49), specifically from dietary sources such as the Mediterranean diet (9). Likewise with the knowledge of nitrate and nitrite being a source of nitric oxide in the body, commercial companies (53) have been promoting the use of inorganic nitrate

products (i.e., beetroot juice) as a means of improving exercise endurance via increased oxygen supply (16, 34, 36, 54). The benefits of nitrate supplementation for exercise have however been inconclusive as some studies found no improvement after nitrate supplementation (37, 38, 55). This study therefore aimed to measure the concentration of nitric oxide in the body of healthy participants after the ingestion of inorganic nitrate. This literature review will describe topics around the production of nitric oxide, methods used to detect nitric oxide in the body and factors that affect nitric oxide concentration in the body.

## 2.2 Nitric oxide production

Nitric oxide is a messenger molecule that functions in vasodilation, blood flow regulation, regulation of platelet aggregation as well as neurotransmission. Two different pathways for nitric oxide production have been identified, the endogenous pathway (3, 11) that involves enzymes and the exogenous pathway that involves reduction of dietary nitrates (12).

### *2.2.1 Enzymatic production of nitric oxide*

Förstermann and Sessa (56) described the different isoforms of nitric oxide synthase (NOS) enzyme: endothelial NOS (eNOS or NOS III), neuronal NOS (nNOS or NOS I) and inducible NOS (iNOS or NOS II). Both eNOS and nNOS are constitutive as well as calcium/calmodulin-dependent enzymes (56). Inducible NOS is independent of calcium and its expression is up-regulated during inflammatory conditions, due to stimulation by pro-inflammatory mediators (i.e., tumour necrosis factor-alpha, interferon-gamma and interleukin), however it is constitutively expressed in the respiratory tract (57).

The NOS enzymes catalyse the reaction of L-arginine with molecular oxygen as well as with the involvement of cofactors (NADPH, $BH_4$ and FAD) that act as electron donors to produce L-citrulline and nitric oxide (58). Murad (59) then proposed nitric oxide (initially known as endothelial-derived relaxant factor), which is produced in the endothelium, diffuses into smooth muscle cells. In the smooth muscle it binds to haem proteins within soluble guanylyl cyclase (sGC), which activates guanylate cyclase causing an increase in cyclic guanosine monophosphate (cGMP). Cyclic GMP in muscle cells activates protein kinase that leads to the de-phosphorylation of myosin light chains and ultimately smooth muscle relaxation (59).

### *2.2.2 Enzyme-independent production of nitric oxide*

Thereafter, Zweier et al.(48) and Lundberg and Govoni (60) described an alternative pathway for nitric oxide production that does not involve enzymatic action, where nitric oxide metabolites (i.e., nitrate and nitrite) are converted to nitric oxide through reduction. The alternative pathway involves the reduction of inorganic nitrate to nitrite by nitrate reductase bacteria found in the oral cavity (15, 61, 62). Inorganic nitrates are found in green leafy vegetables, beetroot and cured meats (8, 63). The nitrate content in different vegetables is classified in Table 1(63).

Table 1. Vegetables classified according to nitrate content

| Nitrate content (mg/100 g fresh weight) | Vegetable varieties |
|---|---|
| Very low: < 20 | Artichoke, asparagus, broad bean, eggplant, garlic, onion, green bean, mushroom, pea, pepper, potato, summer squash, sweet potato, tomato, watermelon |
| Low: 20 to <50 | Broccoli, carrot, cauliflower, cucumber, pumpkin, chicory |
| Middle: 50 to <100 | Cabbage, dill, turnip, savoy cabbage |
| High: 100 to <250 | Celeriac, Chinese cabbage, endive, fennel, kohlrabi, leek, parsley |
| Very high: >250 | Celery, cress, chervil, lettuce, red beetroot, spinach rocket (rucola) |

Some of the nitrite is reduced to nitric oxide by acid-generating plaque bacteria in the mouth and the remaining nitrite is swallowed (15). In the stomach the acidic pH converts nitrite to nitrous acid, which spontaneously degrades to nitric oxide and other nitrogen species (Figure 1) (12). Production of nitric oxide via the alternative pathway is further stimulated by hypoxic conditions (13).

The elucidation of the alternative pathway had promoted researchers to study the possible benefits of a nitrate-rich diet in the cardiovascular system and in exercise performance. Numerous studies have shown that blood pressure decreases after nitrate ingestion (26, 31, 33, 49, 64-66). The reduction in blood pressure was hypothesised to be attributed to the increase in nitrite from the reduction of the ingested nitrate. Nitrite was found to potentiate vasodilation upon its reduction to nitric oxide, specifically under hypoxic conditions (51). Hunter et al. (13) built on the knowledge of nitrite reduction to nitric oxide by deoxyhaemoglobin. They showed that inhaled nebulised nitrite decreased both pulmonary artery pressure and resistance in hypoxic lambs, which was associated with an increase in exhaled nitric oxide (13).

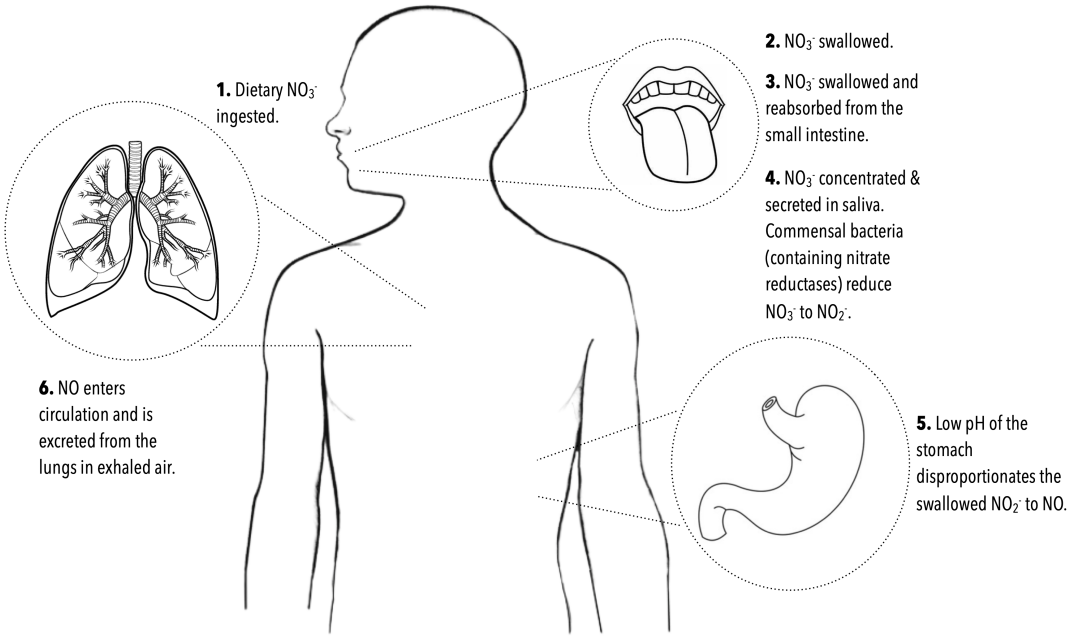


Figure 1. Alternative pathway for nitric oxide (NO) production after ingestion and reduction of nitrate ($NO_3^-$) followed by reduction of nitrite ($NO_2^-$) (adapted from Webb et al. (33)).

## 2.3 The effect of nitric oxide and its metabolites on health

Earlier studies (67, 68) reported that nitrate could be detrimental to human health, as ingestion of nitrate-rich foods or drinking-water (containing nitrate amongst other ions) would be reduced to nitrite in the body. Nitrite produced from the reduction of nitrate was found to have toxic effects as it would react with oxygenated haemoglobin to form methaemoglobin and nitrate (68). Excess methaemoglobin (i.e., methaemoglobinaemia) decreases oxygen delivery around the body as methaemoglobin lacks oxygen-carrying capacity. Methaemoglobinaemia is specifically fatal in infants and commonly results in a condition known as 'Blue baby syndrome' (68, 69).

Moreover, Rogers et al. (70) and Badawi et al. (71) associated the nitrite formed from the reduction of dietary nitrate with increased risk of developing oral cancer. Rogers et al. (70) stated that nitrate in itself was not associated to increased risk of oral cancer,

but rather found a relationship between fruits rich in nitrate and decreased risk of cancer. They further elaborated that nitrate was a substrate for nitrite which may bind to amines or amides to form nitrosamines linked to an increased risk of cancer. Likewise, Badawi et al. (71) reported higher levels of nitrate and nitrite as well as nitrate reductases in cancer patients compared to the healthy individuals. Although increased nitrate levels are most likely due to increased dietary nitrate consumption, Badawi et al. (71) argued that this increases the availability of nitrite to react with nitroso species, which may be linked to carcinogenesis.

However, in later studies, Webb et al. (33) and Jonvik et al. (49) reported beneficial effects of nitrate, which is reduced to nitrite and subsequently to nitric oxide. Both studies concluded that dietary nitrates (specifically in beetroot and other nitrate-rich vegetables) lowered arterial blood pressure. They attributed the decreased blood pressure to the higher availability of nitric oxide from the nitrate-nitrite-nitric oxide production pathway. Nitric oxide, as a potent vasodilator, would then act to dilate blood vessels leading to decreased blood pressure (51). The realisation of nitrate being an alternative source of nitric oxide then instigated the research around nitrate-rich diets and their roles in cardiovascular health (31, 33).

Following the results of dietary nitrate lowering blood pressure and increasing blood flow, Bailey et al. (34) and Rasica et al. (36) investigated the possible benefits of dietary nitrate on exercise performance. Both studies associated nitrate supplementation with a reduction in oxygen consumption during exercise, which increased exercise endurance (34, 36). While other studies observed no significant impact on exercise after nitrate ingestion, which may be due to differing dietary nitrate concentrations administered among other factors (37, 38, 55). The inconsistencies in the results around the impact of dietary nitrate on exercise may be due to a lack of

understanding around the non-enzymatic pathway of nitric oxide production. Researchers mainly focused on the measurement of nitrate and nitrite in the circulation of individuals after nitrate ingestion (34, 72).

### 2.4 Nitric oxide measurement techniques

Nitric oxide production can be measured directly or indirectly in the body. Electrochemical detection of exhaled nitric oxide is one of the techniques used, which converts the gas sample into an electrical signal proportional to the concentration of the gas in the sample (Table 1) (73). This is applicable especially for gases that can be electrochemically oxidised or reduced (74). This method is cheaper and has also been miniaturised and made portable to allow for clinical use on both children and adults (74). However, it is not as sensitive to nitric oxide detection as other nitrogen species may be interpreted falsely as nitric oxide (75). Portable electrochemical detectors, such as the NObreath (Bedfont Scientific Ltd, Kent, UK), were shown to have a large variation in two repeated measures and therefore recommendations were made to obtain at least three exhalation measures from each individual being sampled (76, 77). Electrochemical detection consists of sensors that measure exhaled nitric oxide at a fixed flow rate. Most electrochemical detectors are pre-calibrated at the manufacturers and thus do not require frequent calibration compared to chemiluminescence devices that are routinely calibrated. Although a pre-calibrated analysers may be preferable to many users, the lack of frequent calibration may eventually lead to changes in the performance of the analyser (73).

Alternatively, chemiluminescence is a technique that is commonly used for the detection of nitric oxide in a laboratory setting (Table 2). Despite the high cost, bulkiness of the equipment and its restriction to laboratory use only, this technique

allows for the direct measurement of nitric oxide and its metabolites (i.e., nitrite and nitrate), both in real time and at a later stage (74, 78). Chemiluminescence is also highly sensitive to nitric oxide detection and is capable of differentiating between nitric oxide and other nitrogen species (79). Bates (79) and Maniscalco et al. (74) describe the chemiluminescence method at length, however in summary this method requires an oxygen source to generate ozone, which reacts with nitric oxide in the sample to form unstable nitrogen dioxide ($NO_2$*). As the excited $NO_2$* molecule reverts to its ground state ($NO_2$) photons are emitted. The photons are then detected and amplified by a photomultiplier tube (PMT), which generates a signal that is proportional to the nitric oxide concentration within the sample. Nitric oxide concentration is measured in parts per billion (ppb) or in parts per million (ppm) (79). Ozone-based chemiluminescence is the gold standard for measuring nitric oxide and its metabolites (74, 80).

Antus et al. (76) compared both detection methods and reported that the electrochemical detector recorded marginally higher exhaled nitric oxide values compared to those recorded by a chemiluminescence analyser. Despite this observation the study also found a strong correlation between exhaled nitric oxide recorded from both detection methods.

Table 2. Differences between chemiluminescence and electrochemical detection

| **Chemiluminescence detection** | **Electrochemical detection** |
|---|---|
| "Gold standard" for gaseous NO (nitric oxide) detection. Laboratory use only. | More recently employed to measure exhaled NO. Clinical and home-based use. |
| ***Principle:*** | |
| Gaseous NO molecules are detected via electromagnetic radiation. | Gaseous NO is converted into an electrical signal. |

| | |
|---|---|
| The electromagnetic radiation, in the form of photons, are detected and amplified by a photomultiplier tube (PMT) in the analyser. The signal manifested by the PMT is directly proportional to nitric oxide concentration in the sample. | The electrical signal is caused by a chemical reaction of nitric oxide in a sensor within the analyser. The chemical reaction produces a measurable change in the form of an electrical signal proportional to nitric oxide concentration in the sample. |
| ***Sensitivity:*** | |
| Highly sensitive with a detection threshold of one part per billion (ppb), measurement range: <1-500 000 ppb | High sensitivity with detection limit of ~ 1 ppb, measurement range: <1-600 ppb |
| ***Weight:*** | |
| 16 kg | ~0.4 kg |
| ***Calibration:*** | |
| Calibration should be done before measurements are carried out. Two-step calibration with a zero-air filter and with a known concentration of nitric oxide gas. | Calibrated at the manufacturer. |
| ***Required appendages:*** | |
| Requires an external oxygen source (tank) as well as a vacuum pump and an external inert (He, $N_2$, Ar) gas tank. | None |
| ***Cost:*** | |
| ~500 000 nad | ~80 000 nad |

## 2.5 Factors that affect exhaled nitric oxide

### *2.5.1 Expiratory flow rate*

The European Respiratory Society (ERS) and the American Thoracic Society (ATS) published recommendations with the aim of standardising measurement of exhaled nitric oxide (17). A single-breath exhalation was made the using the common breathing manoeuvre whereby the participant exhales slowly to vital capacity at a specific flow

rate either online, directly into the analyser, or offline, into a reservoir (i.e., a 12 litre Mylar balloon) that can be analysed at a later period. Studies have shown there are various factors that affect the concentration of nitric oxide in a sampling breath, one of the main factors being flow rate of the exhalation (24, 81). The ATS and ERS recommended a flow rate of 50 mL/s when measuring exhaled nitric oxide. This flow rate was chosen as a rational compromise between sensitivity of measurement and comfort of the participant (17).

Studies have observed differences between participants with asthma and normal participants, using different flow rates when exhaling (4, 5, 18, 25, 82). Differing flow rates have also been shown to measure nitric oxide from different sites of the lower respiratory tract (25). Low flow rates (e.g., ≤50 mL/s) are linked to nitric oxide from the trachea, while higher flow rates (e.g., ≥200 mL/s) are used to measure nitric oxide from the alveoli. However, Eckel and Salam (83) argue that a single high flow rate is not an accurate representation of nitric oxide from the alveoli, but rather studies should use multiple, differing flow rates to accurately distinguish between alveolar and tracheal nitric oxide production. An earlier study measured exhaled nitric oxide at five different flow rates to determine the amount of exhaled nitric oxide contributed from the upper (i.e., nasopharynx) airways compared to the lower (i.e., tracheobronchial tree) airways (84). They discovered that nitric oxide could be detected in the lower airways at higher flow rates (≥ 200 mL/s) due to decreased time for diffusion of nitric oxide into the airways, whereas lower flow rates detect nitric oxide from the upper (bronchial) airway as there is more time for diffusion to occur (84).

### *2.5.2 Respiratory conditions*

Nitric oxide is produced in the body under normal physiological conditions from constitutive endothelial NOS enzymes catalysing reactions between L-arginine and

oxygen molecules (85). Gustafsson et al. (85) then demonstrated the excretion of endogenous nitric oxide into exhaled air in rabbits and humans. The study further confirmed that nitric oxide was produced from L-arginine by intravenously administering NOS inhibitors ($N^G$-monomethyl-L-arginine; L-NMMA or $N^w$-nitro-L-arginine methyl ester; L-NAME) in rabbits, which resulted in an attenuation of nitric oxide in the expired air of rabbits (85). This finding was also confirmed by Lundberg et al. (86) who measured nitric oxide from the sinuses and nasal passages of healthy individuals. They found that the human sinuses contained a large amount of nitric oxide. Lundberg et al. (86) further administered NOS inhibitor L-NAME into the nasal sinuses of the individuals, which resulted in decreased nitric oxide concentration in their sinuses. The study also performed immunohistochemical staining and mRNA hybridisation of the nasal sinuses and nasal epithelium in patients (being treated for proptosis) and found large amounts of iNOS in sinus epithelial cells and less so in the nasal epithelium (86). Another study demonstrated that iNOS in the airways is expressed under inflammatory conditions where asthmatic individuals displayed strong immuno-reactivity for iNOS in their bronchial biopsies, compared to the biopsies taken from healthy individuals that had little to no immuno-reactivity (87).

Studies listed in Table 3 demonstrate that individuals with respiratory conditions have altered exhaled nitric oxide levels compared to healthy individuals. Generally, individuals with inflammatory conditions were observed to have higher exhaled nitric oxide concentrations compared to healthy individuals (Table 3) except for individuals with cystic fibrosis who exhibited low concentrations of exhaled nitric oxide (88-91). Inflammatory lung conditions were hypothesised to increase exhaled nitric oxide due to the induction of iNOS by cytokines and other inflammatory mediators (92). Likewise, studies also observed that patients with controlled asthma (i.e., using inhaled

corticosteroid treatment) have exhaled nitric oxide concentrations similar to healthy participants (Table 3). Similarly, Ho et al. (90) reported no difference in nitric oxide concentrations measured in cystic fibrosis and bronchiectasis patients compared to healthy participants. However, Högman et al. (2) argued that a higher flow rate accounted for the low levels of exhaled nitric oxide observed in the study done by Ho et al. (90). Lim et al. (91) also reported decreased exhaled nitric oxide concentrations in patients with cystic fibrosis and likewise the study used an exhalation manoeuvre at a high flow rate. More recently, Kerley et al. (93) tested the effect of dietary nitrate on exhaled nitric oxide in a cystic fibrosis patient and reported a significant increase in exhaled nitric oxide post-ingestion. However the study was only conducted on one patient and the flow rate at which the exhaled nitric oxide was measured was not indicated (93). Despite the number of studies observing elevated levels of exhaled nitric oxide in individuals with inflammatory conditions, numerous other studies have shown conflicting results. Overall, clarification might be achieved with further studies around exhaled nitric oxide levels in the normal, healthy lung.

Rhinitis is a common respiratory inflammatory disorder, which affects an estimated 25% of individuals worldwide (94, 95). Rhinitis is described as inflammation of the epithelial lining of the nasal cavity. The inflammation is caused by reactions to allergens in the environment which include pollen, dust and particulate matter (96, 97). The burden of particulate matter is especially high in developing countries that do not have measures in place to monitor air quality and pollution levels (96).

Rhinitis can be categorised into two main types: allergic rhinitis and non-allergic rhinitis (94, 98). Individuals with non-allergic rhinitis do not have allergic reactions, but still exhibit nasal symptoms (98) whereas those with allergic rhinitis have allergies accompanied by increased immunoglobulin E (IgE) levels or have a positive reaction

to a skin prick test (99). The manifestations of allergic rhinitis include rhinorrhoea, nasal congestion, sneezing, itchiness and watering of the eyes (95, 99). Mølgaard et al. (98) compared the clinical and demographic characteristics between individuals with allergic rhinitis and those with non-allergic rhinitis. They however found that both individuals with non-allergic and those with allergic rhinitis all exhibit symptoms, which are more persistent in the individuals with non-allergic rhinitis. Mølgaard et al. (98) further stated that individuals with allergic rhinitis exhibit more sneezing and itchiness of the eyes during allergy-prone seasons compared to individuals with non-allergic rhinitis.

Allergic rhinitis is brought on by allergens activating mucosal mast cells (97) leading to the production of IgE as well as the synthesis of eosinophils, basophils and other inflammatory cells (99). The IgE binds to the membranes of mast cells and basophils, which accumulate in the nasal mucosa and cause the release of inflammatory mediators such as histamine and leukotrienes, which in turn provoke the allergic response (100). The inflammatory process then stimulates iNOS that is contained in the epithelial lining of the respiratory tract. The up-regulation of iNOS subsequently increases the concentration of nasal nitric oxide (57, 101).

Generally, antibody testing (i.e., IgE tests) and skin prick testing are used to diagnose rhinitis (94, 99). However, as the tests may be scarce, unavailable or costly in certain settings, researchers have explored the use of nitric oxide monitoring to diagnose rhinitis (94). Exhaled nitric oxide can be measured using nitric oxide analysers that can detect nitric oxide concentrations when individuals breathe into the analyser. Although exhaled nitric oxide measurements require machines that are costly, the development of portable nitric oxide analysers have become beneficial in clinical settings (102).

The data around exhaled nitric oxide in individuals with rhinitis is mostly consistent, where numerous studies (95, 101, 103-105) agree that individuals with allergic rhinitis have higher concentrations of nasal exhaled nitric oxide compared to healthy individuals. While other studies (106-108) observed no difference in exhaled nitric oxide levels between individuals with rhinitis and healthy individuals. It was then postulated that the variation of results from the different studies may be due to differences in methodology, including the selection of the study population (109). Differences in exhaled nitric oxide were specifically found between persons with allergic rhinitis and those with non-allergic rhinitis, where individuals with non-allergic rhinitis had exhaled nitric oxide levels comparable to healthy controls (105).

Moreover, asthma is another common respiratory condition that is prevalent world-wide (110) with a rising occurrence in Africa, but the statistics may still be underestimated due to the lack of data (111). A high prevalence of asthma was also observed in Namibia, specifically in the urban areas (96), which they associated with uncontrolled levels of particulate matter in suburban areas. Although Hamatui & Beynon (96) had a small study population, their findings were supported by Halonen et al. (112) who correlated increased hospital visits due to asthma in children with periods where small and traffic-linked particulate matter as well as gaseous air pollutants were increased.

Asthma is a reversible inflammatory condition of the respiratory airways and is characterised as bronchial hyper-responsiveness and the presence of IgE in the circulation (82, 113). The inflammatory process observed in asthma is usually brought on by environmental allergens, but it can also be triggered by other factors such as exercise, infections, drugs and weather conditions (97). Individuals with asthma undergo airway remodelling, which includes thickening of the bronchial tract as a

reaction to allergens or other inflammatory causative agents (114). The immune cells, specifically mast cells in individuals with asthma produce numerous inflammatory cytokines (115).

Nitric oxide in the respiratory tract is significantly increased by inflammatory conditions including asthma as observed by numerous studies (Table 2). The increase in exhaled nitric oxide has been attributed to the increased iNOS expression brought on by the inflammatory process in asthmatic individuals (82, 87). Roos et al. (82) also observed increased exhaled nitric oxide in asthmatics after allergen provocation compared to unprovoked asthmatics, which correlated with iNOS expression in the airway epithelium. Exhaled nitric oxide thus became a marker for inflammation in the respiratory tract as studies demonstrated that gaseous nitric oxide is increased in inflammatory conditions (4, 5). As exhaled nitric oxide measurement became more widely available in various countries, the measurement has become an adjunct when testing diagnosed asthmatics or suspected asthmatics (113). Although exhaled nitric oxide is not a diagnostic tool, it has become a beneficial measurement for monitoring disease activity, especially in asthma (91).

Table 3. Measurement of exhaled nitric oxide in healthy participants and participants with respiratory inflammation

| **Author (reference No.)** | **No. of participants** | **Participant Gender** | **Study group(s)** | **Methods** | **Results** |
|---|---|---|---|---|---|
| Kharitonov et al. (5) | 180 | 116 males<br>86 females | 67 healthy controls<br>61 untreated asthmatics<br>52 treated asthmatics | Chemiluminescence analyser<br>Slow vital capacity breath manoeuvre | Average peak eNO[a] in controls was 80.2±4.1 ppb<br><br>Average peak eNO in untreated asthmatics was 283±16 ppb<br><br>Average peak eNO in treated asthmatics was 101±7 ppb<br><br>eNO was significantly higher in untreated asthmatics compared to controls and treated asthmatics |
| Alving et al. (4) | 25 | Not specified | 12 controls (3 with LRTI[b])<br>8 asthmatics<br>5 intubated patients (with no known asthma) | Chemiluminescence analyser<br>Normal tidal breathing | In controls: nasal eNO was 23±2 ppb and oral eNO was 9±1 ppb<br><br>Controls eNO ranged between 5-16 ppb and asthmatic eNO ranged between 21-31 ppb<br><br>Intubated patients' eNO was ≤3 ppb<br><br>Controls with LRTI had average eNO of 32±4 ppb |
| Kharitonov et al. (40) | 18 | 11 males<br>7 females | All with URTI[c] | Chemiluminescence analyser<br>Slow vital capacity breath manoeuvre | During symptomatic URTI eNO was 315±57 ppb<br><br>After recovery from URTI eNO was 87±9 ppb |

| Author (reference No.) | No. of participants | Participant Gender | Study group(s) | Methods | Results |
|---|---|---|---|---|---|
| Baraldi et al. (116) | 94 (children) | 70 males<br>24 females | 47 asthmatics<br>47 healthy controls | Chemiluminescence analyser<br>Normal tidal breathing | Average (after NO-free inhalation) eNO in asthmatics was 23.7±1.4 ppb (range: 8-60 ppb)<br><br>Average eNO in healthy controls was 8.7±0.4 ppb (range: 3.5-15 ppb)<br><br>Average (after ambient air inhalation) eNO in asthmatics was 49±4.6 ppb (range:14-200 ppb) |
| Saito et al. (117) | 65 | Not specified | 50 asthmatics (28 uncontrolled asthma and 22 controlled asthma)<br>15 healthy controls | Electrochemical detection | Average $FeNO^{d}$ for healthy controls was 6.05 ppb (range: 5.19-6.90 ppb)<br><br>Average FeNO for uncontrolled asthmatic group was 15.6 ppb (range: 12.5-18.7 ppb)<br><br>Average FeNO for controlled asthmatic group was 8.18 ppb (range: 6.69-9.67 ppb) |
| Persson et al. (118) | 60 | 21 males<br>39 females | 34 asthmatics (23 controlled asthma)<br>20 healthy controls<br>6 smokers | Chemiluminescence analyser<br>Normal tidal breathing and 15-second breath holding manoeuvre | Controls' eNO was 8.4 (SE 1.2) ppb<br><br>Asthmatics' eNO was 10.3 (3.3) ppb<br><br>Smokers' eNO was 3.9 (1.0) ppb |

| Author (reference No.) | No. of participants | Participant Gender | Study group(s) | Methods | Results |
|---|---|---|---|---|---|
| Lehtimäki et al. (119) | 32 | 10 males<br>22 females | 16 asthmatics<br>16 healthy controls | Chemiluminescence analyser<br>at three different exhalation flow rates | Bronchial NO flux in controls was 0.7±0.1 nL/s<br>Bronchial NO flux in asthmatic patients was 3.6±0.4 nL/s<br><br>Alveolar NO concentration was 1.0±0.2 ppb in controls<br>Alveolar NO concentration was 1.2±0.5 ppb in asthmatic patients<br><br>Bronchial NO flux in asthmatics, after treatment, reduced to 0.7±0.1 nL/s |
| Lim et al. (91) | 45 | 23 males<br>22 females | All with cystic fibrosis (CF)<br>(9 CF patients with high risk of allergic bronchopulmonary aspergillosis (ABPA) & 36 CF patients with low risk of ABPA | Chemiluminescence analyser<br>Three separate exhalations at 200mL/s | Median eNO of 1.8 (range 1.4-3.2) ppb in CF patients at high risk of ABPA<br>Median eNO of 2.9 (range 1.2-18.0) ppb in CF patients at low risk of ABPA<br><br>eNO of 2.0 ppb in CF patients at high-risk of ABPA (taking oral glucocorticoids)<br>eNO of 3.1 ppb in CF patients at low-risk of ABPA (taking glucocorticoids) |
| Grasemann et al. (89) | 15 | 6 males<br>9 females | Cystic fibrosis patient eligible for treatment | Electrochemical detection | FeNO of 8.5±5.0 ppb before treatment<br>FeNO of 16.2±15.5 ppb after treatment |

| Author (reference No.) | No. of participants | Participant Gender | Study group(s) | Methods | Results |
|---|---|---|---|---|---|
| Ho et al. (90) | 108 | Not specified | 36 patients with cystic fibrosis<br>16 patients with bronchiectasis<br>34 patients with asthma<br>22 healthy controls | Chemiluminescence analyser<br>Slow vital capacity manoeuvre | Median eNO of 4.4 (1.0-7.8) ppb in healthy controls<br>Median eNO of 4.0 (2.8-7.2) ppb in CF patients<br>Median eNO of 10.1 (5.8-13.3) ppb in asthmatic on corticosteroids<br>Median eNO of 10.4 (7.3-29.4) ppb in steroid-naive asthmatics<br>Median eNO of 6.7 (4.9-7.3) ppb in bronchiectasis on corticosteroids<br>Median eNO of 4.7 (4.4-7.8) ppb in steroid-naive bronchiectasis |

[a]Exhaled nitric oxide concentration in parts per billion (ppb)

[b]Lower respiratory tract infection

[c]Upper respiratory tract infection

[d]Fractional exhaled nitric oxide

### *2.5.3 Dietary metabolites*

Certain dietary components have been shown to influence nitric oxide levels in the body, which can be subsequently measured in exhaled air. The main dietary components being nitrate and nitrite (Table 1) of which nitrate has high concentrations in numerous root vegetables and green leafy vegetables, while nitrite is used for its preservative and curing properties on meat products (63, 120). Researchers established an alternative pathway, which was designated as the entero-salivary pathway that describes dietary nitrate reduction to nitrite and eventually to nitric oxide and other nitrogen species (121). After the discovery of the alternative nitric oxide production

pathway a few researchers have investigated the impact of nitrate intake on nitric oxide in the airways (20, 22, 28, 30, 93, 122-126).

On the one hand most of the studies were conducted on healthy participants provided with a dietary nitrate intervention in the form of a nitrate-rich meal (20, 22, 122), nitrate juice formulation (123, 124, 126), or nitrate solution (nitrate solute dissolved in water) (30, 122, 127). While on the other hand, some of the studies involved patients with a pathophysiological condition affecting their airways (28, 93, 125, 126). Despite the differences in the study groups, the outcomes of all the studies agree that nitrate intake increases exhaled nitric oxide in the participants. The increase in exhaled nitric oxide after nitrate intake, despite the respiratory condition of the participants, emphasised the production of nitric oxide independent of the enzymatic action of nitric oxide synthase.

Furthermore, studies either employed electrochemical detection or chemiluminescence as the technique to measure nitric oxide. Both techniques were determined to produce correlating measurements of exhaled nitric oxide (128). Most of the studies recorded maximal increases in exhaled nitric oxide approximately one hour to two hours after nitrate-intake(20, 22, 30, 93, 122, 124, 127). The differences in nitrate intervention i.e., dissolved nitrate solution, beetroot juice, or nitrate-rich meal, used in the cited studies did not appear to impact the nitrate-nitrite-nitric oxide pathway in the body. There was however a significantly higher percent increase in exhaled nitric oxide in studies that had longer intervention periods (28, 123, 125).

### *2.5.4 Oral bacteria*

Initially it was thought that the human body was incapable of reducing nitrate to nitrite, as it lacked the necessary enzymes (62). However, bacteria that inhabit the human oral

cavity on the surface of the posterior-end of the tongue contain enzymes with nitrate reductive properties (15, 61, 129). Tannenbaum et al. (61) demonstrated an increase in nitrite concentration in human saliva after the consumption of inorganic nitrate (i.e., celery juice). This study had also confirmed this observation by blocking the oral bacterial action with the use of bactericidal mouthwash. They reported no increase in salivary nitrite concentrations after ingestion of inorganic nitrate as a result of the bactericidal mouthwash. Tannenbaum et al. (61) quantified nitrite concentration using the Griess assay, which was not the most sensitive method to quantify nitric oxide and its metabolites (130, 131).

A subsequent study confirmed the observation of nitrate reduction to nitrite, specifically on the posterior region of the tongue in rats (15). Duncan et al. (15) demonstrated nitrate reduction to nitrite on the surface of rat tongues that were incubated with a known concentration of potassium nitrate. The study also illustrated an abundance of Gram-positive and Gram-negative bacteria on the posterior surface of rat tongues compared to the anterior surface. Thereafter, Duncan et al. (15) also performed the same experiment on rats grown in a germ-free environment and found no nitrite production after incubating the rat tongues with potassium nitrate.

In addition to nitrite quantification, Duncan et al. (15) also measured nitric oxide production in the mouth after nitrate ingestion and noted a significant increase in both nitric oxide and nitrite after nitrate ingestion. This study also used the Griess assay to quantify nitrite concentrations. Nonetheless, Duncan et al. (15) carried out a separate experiment in which they measured the production of nitric oxide from the mouth in healthy humans. The participants each ingested a solution of potassium nitrate after which air was sampled from the mouth of the participants for measurement of nitric oxide using chemiluminescence. There was an increase in nitric oxide, which was

comparable to the increase in nitrite over time (15). They also observed the effects of antibacterial mouthwash in healthy participants. The antibacterial mouthwash caused an initial decrease in oral nitric oxide before returning to baseline ten minutes later. These findings are indicative of the impact of oral hygiene on the nitric oxide production and have also confirmed that nitric oxide production can occur in the oral cavity (15, 132). A study done by Kamimura et al. (132) measured exhaled nitric oxide before and after oral care (i.e., brushing of teeth followed by gargling with water) in which they found decreased nitric oxide levels after oral care, which they assumed was due to oral bacteria and dental plaque removal (and the subsequent decrease in oral acidity).

### *2.5.5 Acidic environment*

An accidental discovery by Furchgott (133) highlighted that an acidic environment is essential for formation of nitric oxide from inorganic nitrite. Nitrite, produced from the reduction of nitrate in the oral cavity, is swallowed and disproportionated, by the acidic environment of the stomach, to yield nitric oxide and other nitrogen species (29, 134, 135). Lundberg et al. (29) modulated the pH of the stomach by administering proton pump inhibitors to individuals and measured nitric oxide gas regurgitated from the stomach. They found that regurgitated nitric oxide levels were significantly reduced in individuals treated with proton pump inhibitors compared to individuals who were not treated with proton pump inhibitors (29). Furthermore, the study demonstrated an increase in *in vitro* nitric oxide production from a dietary nitrate source placed in hydrochloric acid, which was then attenuated when a basic solution of carbonated water was added (29). Although Lundberg et al. (29) had a small sample size, they reported significant results for nitric oxide production in acidic conditions. Another study demonstrated similar results after observing the effects of ascorbic acid

mouthwash before nitrate intake in healthy participants (Duncan et al., 1995). The study showed an increase in nitric oxide almost immediately after nitrate intake subsequent to the ascorbic acid mouthwash.

Subsequently, Carlsson et al. (135) used the concept of nitrite disproportionation to measure nitric oxide production in acidified urine samples at different pH levels. They observed increased production of nitric oxide from nitrite-containing urine that was acidified, which was further enhanced by the addition of ascorbic acid. The results seen in the study compared to previous findings; however *in vitro* studies may not necessarily yield the same results as *in vivo*.

# Chapter 3

# 3 Research Methods

## 3.1 Research Design

The research was a quantitative study, consisting of observational and experimental aspects. Exhaled nitric oxide from healthy, asthmatic participants and participants with rhinitis was measured at different exhalation flow rates. Additionally, the effects of beetroot juice ingestion on exhaled nitric oxide were studied in healthy participants by modulating their oral bacteria, using antibacterial mouthwash, and stomach pH, using antacids, and thereafter the effects on exhaled nitric oxide were measured. All measurements, excluding the 24-hour experiment that was self-reported by the participants, were conducted by the researcher in the physiology laboratory at the Hage Geingob Campus. The participants with asthma as well as those with rhinitis were recruited for a total of two days, while the healthy controls were recruited for a total of 11 laboratory visits, over a period of eight weeks with a seven-day wash-out period between each beetroot juice experiment.

### 3.1.1 Study population

The target population was all staff (~130) and students (~900) from the Hage Geingob Campus who were healthy, or diagnosed with asthma, or had complaints of seasonal rhinitis. However, due to the COVID-19 pandemic recruitment of individuals with asthma and rhinitis was increased to the general public in the Windhoek area. The participants on campus were recruited using an online learning platform (Moodle), while participants from the general public were recruited using social media platforms and by word of mouth. The participants were recruited and began with experimental procedures between the months of December 2020 and June 2021. All participants who volunteered for the study were screened, using a short questionnaire (Appendix

4) to determine whether they met the inclusion criteria and thereafter were assigned to an appropriate group (i.e., healthy, asthmatic or rhinitis).

### 3.1.2 Inclusion criteria

Participants were required to be between 18-40 years of age, in order to maintain a homogenous sample group.

The following types of participants were included in the study:

- Healthy participants who were in good health i.e., no history of respiratory disease, non-smokers, free of chronic disease and not on any medication except for contraceptive pills.
- Participants with seasonal rhinitis (i.e., with complaints of sneezing, rhinorrhea, congestion, itchy and watery eyes that is not related to a cold or flu), diagnosis of allergic rhinitis was made when history and physical findings were consistent with an allergic cause (e.g., clear rhinorrhea, pale discolouration of nasal mucosa, and red and watery eyes) and one or more of the following symptoms were present: nasal congestion, runny nose, itchy nose, or sneezing.
- Participants diagnosed with asthma (i.e., history of wheezing, diagnosed with having asthma as well as spirometry and peak expiratory flow to validate the condition of the participants).
- Only participants who were fully informed of the protocol and provided written consent for participation were included in the study.
- Female participants were required to have a negative pregnancy test to be included in the study.

### 3.1.3 Exclusion criteria

- Participants with the following criteria were excluded from participating in the study:
- Participants over 40 years of age, due to altered pulmonary physiological state from the age of 40.
- Participants with a history of cardiovascular disease and peripheral vascular disease
- Participants who were taking a course of antibiotic treatment prior to the study.
- Participants who smoke cigarettes or other tobacco products were excluded.
- Participants who had any dental procedure prior to the study.
- Lactating females were excluded from the study as nitrite crosses into breast milk that could cause methaemoglobinaemia in the infant.
- Participants diagnosed with gastro-oesophageal reflux disease (GERD) or with complaints of acid reflux or gastritis.

## 3.2 Sample size determination

The sample size calculations for the groups (i.e., control, asthmatics, and participants with rhinitis) were based on results from studies described in literature. The primary outcome was the increase of nitric oxide concentrations in exhaled air, i.e., measured as the difference between eNO concentrations in healthy individuals compared to those with respiratory inflammation, asthma, or rhinitis. In literature, the average eNO concentration in asthmatic individuals is 40 ppb, with a standard deviation of 30 ppb. The aim of this study was to be able to detect a 25 ppb increase in eNO in asthmatic individuals compared to healthy individuals, with a power of 90% and a significance

level of 5%, thus the estimated sample size was 32 participants diagnosed with asthma; n = 1 + 2(10.51) * (30/25)^2 = 32 , where 10.51 is a constant dependent on the power and the significance level (136).

Additionally, the average eNO concentration in individuals with rhinitis is 200 ppb, with a standard deviation of 50 ppb. This study aimed to be able to detect a difference of 90 ppb between healthy individuals and those with rhinitis. With a power of 90% and a significance level of 5%, the estimated sample size is 7; n = 1 + 2(10.51) * (50/90) ^2 = 7. The sample size for the whole study was therefore a minimum of 30 healthy participants, 32 participants with asthma and 7 participants with rhinitis, thus totalling 69 participants.

## 3.3 Research Instruments

### 3.3.1 Nitric oxide measurement

Chemiluminescence technique (Sievers NOA280i, GE Analytical Instruments, Boulder, CO, USA) was used to measure nitric oxide in the exhaled oral and nasal samples. This technique quantifies exhaled nitric oxide concentration by way of nitric oxide in the sample reacting with ozone produced in the analyser connected to an oxygen source. The reaction between nitric oxide and ozone produces nitrogen dioxide in its excited state and as it converts back to its ground state, photons are emitted. These photons are amplified and detected by a photomultiplier tube, which produces a signal that is proportional to the concentration of nitric oxide (Table 2).

Similarly, electrochemical detection (NOBreath, Bedfont Scientific Ltd., Kent, UK) was also used to directly measure exhaled nitric oxide from oral breath samples. This detector contains a sensor that converts gas concentrations into electrical signals. The detector has a buffer system that captures the last portion of an exhaled sample. This

sample is then transferred to the sensor in the detector, where the nitric oxide gas is subjected to a chemical reaction due to catalytic sensor. A detectable change is emitted via an electrical circuit. This output is measurable and is linearly proportional to the partial pressure (i.e., concentration) of nitric oxide in the sample (Table 2).

### 3.3.2 Physiological measurements

Blood pressure (BP) and heart rate (HR) were measured using an ambulatory blood pressure monitor (Welch Allyn ABPM 7100, New York, USA). The participants were seated upright and rested for 30 minutes before BP and HR were recorded. Pulmonary function was assessed using spirometry (EasyOne® Air, ndd Medizintechnik, Zurich, Switzerland). The parameters that were measured were forced expiratory volume in one-second (FEV1), forced vital capacity (FVC) and peak expiratory flow (PEF). The FEV1/FVC ratio was also determined for each participant.

Each participant's anthropometric and demographic data (i.e., weight, height, age, sex, and race) was recorded before spirometry began. The height was measured in metres, using a stadiometer, with the participants standing barefoot on levelled ground. The weight, in kilograms, was measured using a manual scale. The participants were advised to dress in light clothing and were barefoot when their weight was measured.

## 3.4 Procedure

### 3.4.1 Baseline Measurements

Prior to the experiments, baseline measurements of the parameters described below were determined for each participant.

Firstly, the participants exhaled using the breath manoeuvres described earlier (17). Exhaled nitric oxide measurements were done using the Sievers NOA280i (GE

Analytical Instruments, Boulder, CO, USA) and the NObreath (Bedfont Scientific Ltd, Kent, UK). The Sievers NOA280i was calibrated, before the measurements, according to the manufacturer's instructions, while the NOBreath monitor was pre-calibrated by the manufacturer. The participants performed the breath manoeuvres in triplicate for each different flow rate (30, 50, 150 and 250 mL/s) in order to determine baseline exhaled nitric oxide concentrations from each different respiratory compartment i.e., tracheal and alveolar.

Secondly, the participants were then allowed to rest for 30 minutes before the heart rate and blood pressure was measured (Welch Allyn Ambulatory Blood Pressure Monitor). Lastly, to ensure good respiratory health, lung function tests (i.e., spirometry) were done for each participant.

### 3.4.2 Experiments:

The first part of the experiments sought to determine the different sources of nitric oxide in the respiratory tract, by differentiating between the two respiratory compartments (i.e., tracheal and alveolar). Part two of the protocol recruited 20 healthy participants to determine an optimal dose of nitrate in beetroot juice (Beet It Sport, James White Drinks, Ipswich, UK) that would illicit a significant increase in nitric oxide measured from exhaled breath samples. Part three observed exhaled nitric oxide over a period of 24 hours using a portable nitric oxide analyser (NObreath, Bedfont Scientific Ltd, Kent, UK). Part four determined the effect that oral bacteria had on the production of nitric oxide from dietary nitrate. Lastly, part five observed the effect of stomach pH on the production of nitric oxide after ingestion of beetroot juice. The

timeline of all the experiments that were performed in the study is illustrated in Figure 2.

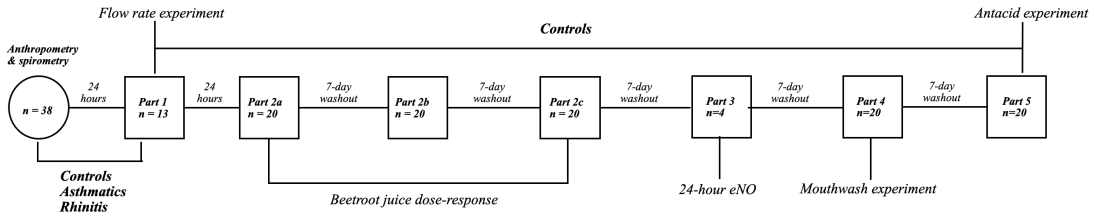


Figure 2. Procedure of the experiments completed on all participants enrolled in the study.

### 3.4.3 Part 1: Differentiation of lower respiratory compartments

To differentiate between the sources of nitric oxide from different respiratory compartments (i.e., tracheal and alveolar), exhaled nitric oxide from healthy controls (n=13) was measured at different flow rates (Table 4). The different flow rates were attained using different expiratory flow restrictors for each different flow rate (GE Analytical Instruments, Boulder, Colorado, USA). These flow restrictors were attached to the breathing apparatus connected to the nitric oxide analyser. Each participant was instructed to perform the exhalation manoeuvre at a constant mouth pressure that was displayed on a computer monitor. The flow rates that were measured are indicated in Table 4. All the participants measured exhaled nitric oxide at different flow rates using chemiluminescence (i.e., Sievers NOA280i, GE Analytical Instruments, Boulder, CO, USA).

Table 4. Measurement of exhaled nitric oxide at different flow rates

| Flow rate (mL/s) | Compartment | eNO (ppb) |
|---|---|---|
| 30 | Tracheal | |
| 50 | | |
| 150 | Alveolar | |
| 250 | | |

Tracheal exhaled nitric oxide was determined as follows:

Each participant wore a nose clip that prevented any nasal breathing. The participants were then instructed to inhale to total lung capacity and exhale immediately into the nitric oxide analyser, at a specified flow rate (i.e., 30 and 50 mL/s). The participants performed the exhalation manoeuvre in triplicate.

Alveolar exhaled nitric oxide was determined as follows:

The participants performed the same exhalation manoeuvres as described for the tracheal exhaled nitric oxide determination. The participants however exhaled at higher flow rates (i.e., 150 and 250 mL/s).

### 3.4.4 Part 2: Dose-response experiment

In order to determine which dose of beetroot juice significantly increased exhaled nitric oxide, healthy controls (n=20) were randomly given a different volume (35, 70, 140 mL) of beetroot juice that contained approximately 200, 400 and 800 mg (137) of nitrate, respectively. Exhaled nitric oxide was measured from each participant before beetroot juice ingestion and then measured again post-ingestion (Figure 3). The participants were asked to come in on three separate days for the different doses (to allow for sufficient washout each visit was separated by a period of seven days). The participants were reminded to avoid nitrate- and nitrite-rich foods as well as other

foods or drinks that would impact nitric oxide concentrations from 24 hours prior to each experiment.

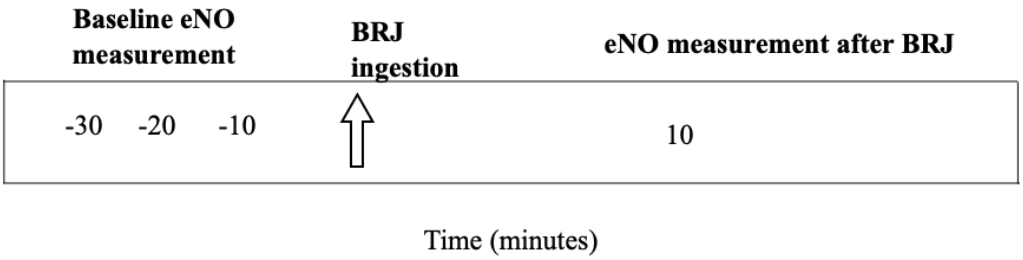


Figure 3. Dose-response experiment. Measurement of exhaled nitric oxide (eNO) pre- and post-ingestion of beetroot juice (BRJ) at different doses (i.e., 200, 400, 800 mg of nitrate).

### 3.4.5 Part 3: Exhaled nitric oxide detection over 24 hours

To determine the pattern of exhaled nitric oxide after ingestion of inorganic nitrate, in the form of beetroot juice (James White Drinks, Ipswich, UK), a small number of controls (n=4) were tasked to measure exhaled nitric oxide using a portable nitric oxide analyser (NObreath, Bedfont Scientific Ltd, Kent, UK) at specified time intervals over a period of 24 hours (Figure 4). The participants were asked to keep a nitrate-free diet, 24-hours prior and during the experiment. Nitrate-rich foods are mainly green leafy vegetables (e.g., spinach, lettuce, cabbage, and rocket) and other root vegetables (e.g., beetroot, radish, turnips, green beans, leeks, spring onion, cucumber, carrot, potato, garlic, and bell peppers). Cured products, such as dried meats (i.e., biltong), bacon and other processed meat, such as cold-cut meats will also be excluded from the diet. Participants were also reminded to avoid caffeine i.e., coffee on the day of the experiment. The participants first measured their exhaled nitric oxide at three different time intervals prior to beetroot juice (70 mL) ingestion. Thereafter, they were instructed to take three repeated measures at each time interval (Figure 4).

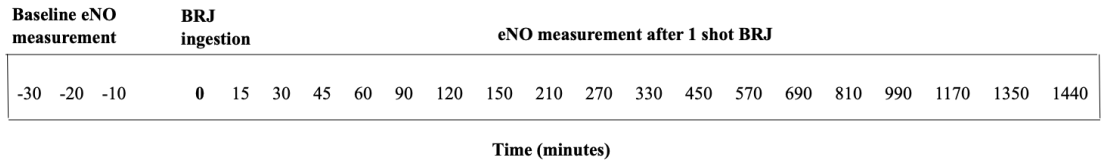


Figure 4. 24-hour exhaled nitric oxide experiment. Measurement of exhaled nitric oxide (eNO) before (30 minutes) and after ingestion of one shot (70 mL) of beetroot juice (BRJ) at different time intervals over a period of 24 hours.

### 3.4.6 Part 4: Oral bacteria modulation

Non-enzymatic nitric oxide formation is also dependent on the presence of bacterial organisms that contain nitrate reductase enzymes that reduce ingested nitrate to nitrite, which is further reduced to nitric oxide. The aim of this experiment was to confirm the role of oral bacteria on the production of nitric oxide.

Exhaled nitric oxide was measured from the control participants (n=20) prior to the experiment. Thereafter, they were instructed to rinse their mouth with 20 mL of antibacterial mouthwash (chlorhexidine gluconate 2 mg/mL, Corsodyl, GlaxoSmithKline Consumer Healthcare, South Africa (Pty) Ltd) for 60 seconds. They were then instructed to ingest 70 mL of beetroot juice. Their exhaled nitric oxide was measured every five minutes after BRJ ingestion, as indicated in figure 5.

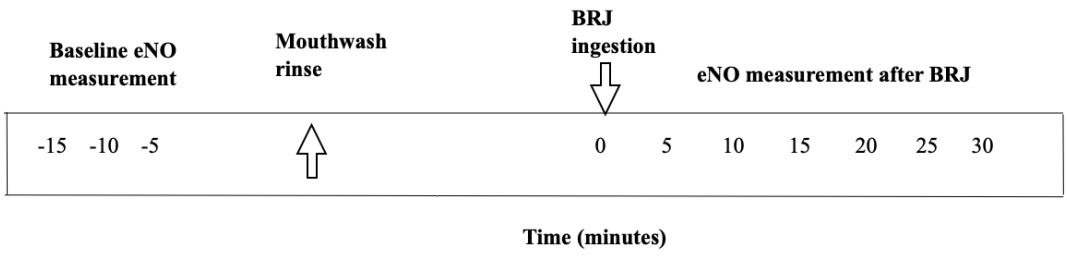


Figure 5. Oral bacteria modulation experiment. Exhaled nitric oxide (eNO) measured before mouthwash rinse and after beetroot juice (BRJ) ingestion

### 3.4.7 Part 5: Stomach pH modulation

Another parameter that is known to affect non-enzymatic nitric oxide production is the presence of a low pH. The low pH, specifically in the stomach, where disproportionation of nitrite to nitric oxide occurs. Therefore, to determine the effect of pH on non-enzymatic production of nitric oxide, firstly eNO was measured from control participants (n=20) prior to the experiment. Thereafter, the controls were instructed to ingest two chewable antacids (Rennie®, Bayer (Pty) Ltd), which was the recommended dose. Their eNO was then measured after ingesting the antacid. After that the participants were instructed to ingest 70 mL of beetroot juice, followed by measurements of their eNO (figure 6).

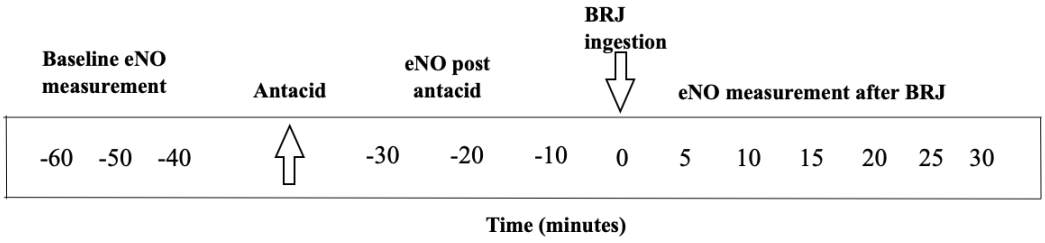


Figure 6. Stomach pH modulation experiment. Exhaled nitric oxide (eNO) before and after administration of two antacid tablets (Rennie® chewable tablets) and post beetroot juice (BRJ) ingestion

All the data collected from each participant was recorded in a data collection sheet (Appendix 4) and then stored securely for data analysis and subsequent archiving.

## 3.5 Data analysis

Exhaled nitric oxide measurements from the NOA 280i analyser were recorded using an analog-digital converter (Powerlab 16/35, ADIntrsuments) and a data collection software (LabChart v8.1.11, ADInstruments). The data was first assessed for normal distribution using the Shapiro-Wilk test. Non-parametric tests were used to analyse data that were not normally distributed. The Kruskal-Wallis test was used to compare

mean eNO concentrations of the controls, asthmatics and participants with rhinitis. Paired comparisons of eNO concentrations were also made using the Friedman test.

Wilcoxon signed-rank test was used to compare exhaled nitric oxide values between two groups in the oral bacteria modulation experiment. A p-value less than 0.05 was considered as significant (GraphPad Prism v8.0.2). All data are presented as means ± standard deviations.

**3.6 Research Ethics**

The study was approved by both the Human Research Ethics Committee, University of Namibia (Appendix 1, Reference number: H-G /569/2020) and the Research Ethics and Coordination division of the Ministry of Health and Social Services (Appendix 2, Ref: 17/3/3/TK). All participants were recruited on a voluntary basis and provided written informed consent before taking part in the study.

There was always a member of the research team available to explain or address any queries that the participants may had. Participants were asked questions to ensure they understood their role in the research prior to consenting (Appendix 3). They were given a copy of the signed consent form and we kept the original copy of the signed consent form.

The participants were allowed to withdraw, without any repercussions, after they had signed the informed consent form.

Participants were not excluded based on their sex, ethnicity or race. Volunteering participants were recruited by word of mouth around the UNAM, Hage Geingob campus and the Windhoek area. This study offered no direct benefit to the participants, but the results from this study may increase the knowledge around the physiological role of dietary nitrate in healthy individuals.

All medical information collected from the participants was kept in a locked file cabinet in the Department of Physiology.

Participants' data was labelled with specific identifiers allowing for confidentiality and only the researchers involved in this study were aware of the participants' true identities. Following data analysis, the data will be stored for a period of five years and will only be accessible by the researchers involved in the study. Thereafter, the data will be archived. The results of this study may be published in an international, peer-reviewed journal.

# Chapter 4

# 4 Results

## 4.1 General characteristics of all participants

Participants were recruited from December 2020 until June 2021. A total of 38 participants were recruited for the study of which seven participants were members of the public. Seven of the participants had rhinitis, six of them were diagnosed with asthma and 25 of them were healthy controls. Two of the controls were excluded, one due to having an upper respiratory tract infection prior to one of the experiments and the other due to non-compliance with the dietary restrictions. All the participants completed a questionnaire to determine their eligibility to the study. Baseline measurements of each participant's physiological parameters i.e., weight, height, body mass index (BMI), blood pressure, lung function and exhaled nitric oxide) were measured on their first visit to the research lab.

The baseline anthropometric and physiological parameters summarised in Table 5 are described using mean±SD. The change in exhaled nitric oxide for each experiment is also presented as mean±SD.

There were no significant differences in the mean age, height, systolic and diastolic blood pressure as well as the heart rate between the control, asthma and rhinitis groups. There was however a significant difference in weight between the participants with rhinitis compared to the control group (59±11 kg and 75±11 kg, p=0.03; Table 5). Accordingly, the BMI was also significantly different between the participants with rhinitis and the control group (21.3±2.76 kg/$m^2$ and 27.7±6.12 kg/$m^2$ p=0.01; Table 5).

Lung function was measured in all participants from each study group. Table 6 describes the summary of the measured lung function test data. There were no differences in lung function i.e., forced vital capacity (FVC), forced expiratory volume

(FEV1) as well as the peak expiratory flow (PEF) between the three study groups. The control group did however demonstrate a higher ratio of the forced expiratory volume in one second to the forced vital capacity (FEV1/FVC) compared to the participants with rhinitis (90.4±5.0% and 84.2±5.7%, p=0.03; Table 6).

Exhaled nitric oxide was measured from each participant enrolled in the study. A comparison of exhaled nitric oxide concentrations was made between the participants who were healthy, participants with asthma and those with rhinitis (Figure 7). The asthmatic participants recorded higher exhaled nitric oxide concentrations compared to the control participants (41±25 ppb vs 12±12 ppb; p=0.04, Figure 1). There was however no significant difference between exhaled nitric oxide measured in the control participants compared to those with rhinitis (12±12 ppb vs 30±31 ppb; p=0.21, Figure 7). The coefficient of variation was used to determine the extent of variation within the study groups. The control group and the group with rhinitis both reported much higher coefficients of variation equal to 104%, while the asthmatic group reported a lower, but still quite large coefficient of variation equal to 61% (Figure 7).

Table 5. Baseline characteristics of all participants

| Characteristics | Control (n=25) | Asthma (n=6) | Rhinitis (n=7) | P-value |
|---|---|---|---|---|
| Age (years) | 24±5 | 21±3 | 23±5 | NS |
| Gender (female/male) | 18/7 | 2/3 | 4/2 | - |
| Weight (kg) | 59±11 | 70±21 | 75±11 | **0.03*** |
| Height (cm) | 166±8 | 164±10 | 165±13 | NS |
| Body mass index (kg/m$^2$) | 21.3±2.76 | 26.1±9.32 | 27.7±6.12 | **0.01*** |
| Systolic blood pressure (mm Hg) | 116±11 | 130±13 | 123±18 | NS |
| Diastolic blood pressure (mm Hg) | 74±8 | 70±11 | 81±10 | NS |
| Heart rate (beats/min) | 84±14 | 75±13 | 88±11 | NS |

One-way ANOVA for comparison of three groups. *Comparison between controls and participants with rhinitis. NS: not significant.

Table 6. Spirometry data for all the participants

| Characteristics | Control (n=25) | Asthma (n=6) | Rhinitis (n=7) | P-value |
|---|---|---|---|---|
| FEV1(L) | 3.0±0.7 | 3.1±0.6 | 3.2±1.0 | NS |
| FVC (L) | 3.3±0.7 | 3.6±0.8 | 3.8±1.0 | NS |
| FEV1/FVC (%) | 90.4±5.0 | 87.5±3.5 | 84.2±5.7 | **0.03*** |
| PEF (L/min) | 469.8±119.3 | 457.4±65.5 | 506.0±168.6 | NS |

One-way ANOVA for comparison of lung function (forced expiratory volume; FEV1, forced vital capacity; FVC, FEV1/FVC and peak expiratory flow; PEF) in three study groups (control, asthma and rhinitis). *Comparison between control and rhinitis. NS: not significant.

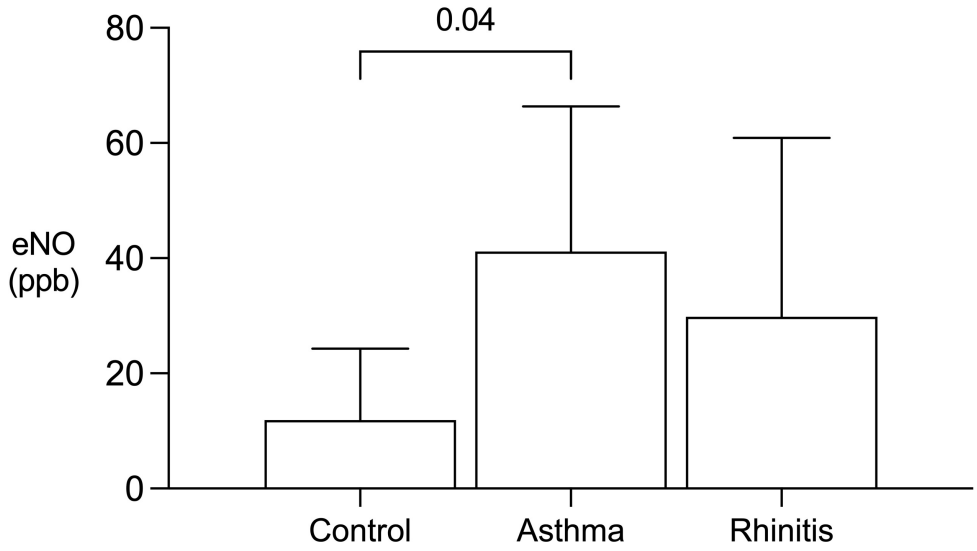


Figure 7. Basal exhaled nitric oxide. Concentrations of exhaled nitric oxide (eNO) measured in control participants (n=25), asthmatics (n=6) and participants with rhinitis (n=7). Kruskal-Wallis test used to compare asthma and rhinitis study groups to the control group. Participants exhaled at 50 mL/s into an electrochemical detector (NOBreath) in triplicate and the average of the three exhaled nitric oxide concentrations was recorded for each participant. Data are presented as mean±SD. ppb: parts per billion

## 4.2 Measurement of exhaled nitric oxide from different respiratory compartments

Exhaling at different flow rates has been used as a proxy for measuring exhaled nitric oxide from different compartments (i.e., tracheal and alveolar) in the airways. Thirteen control participants therefore exhaled at four different flow rates (i.e., 30, 50, 150 and 250 mL/s) to measure exhaled nitric oxide concentrations at each flow rate. There was a general trend for exhaled nitric concentration to decrease as the flow rate increased (Figure 8). With 50 mL/s used as the standardised expiratory flow rate when measuring exhaled nitric oxide, the average exhaled nitric oxide of each other flow rate (30, 150 and 250 mL/s) was compared to the average exhaled nitric oxide at 50 mL/s. There were no significant differences detected between exhaled nitric oxide concentrations at the lower flow rates of 30 mL/s and 50 mL/s (32±29 ppb vs 25±19 ppb, respectively; p=0.52, Figure 8) as well as between 50 mL/s and 150 mL/s (25±19 ppb vs 10±6 ppb,

respectively; p=0.06, Figure 8). The exhaled nitric oxide concentrations were significantly lower when participants exhaled at 250 mL/s compared to 50 mL/s (7±4 ppb vs 25±19 ppb, respectively; p<0.0001, Figure 8).

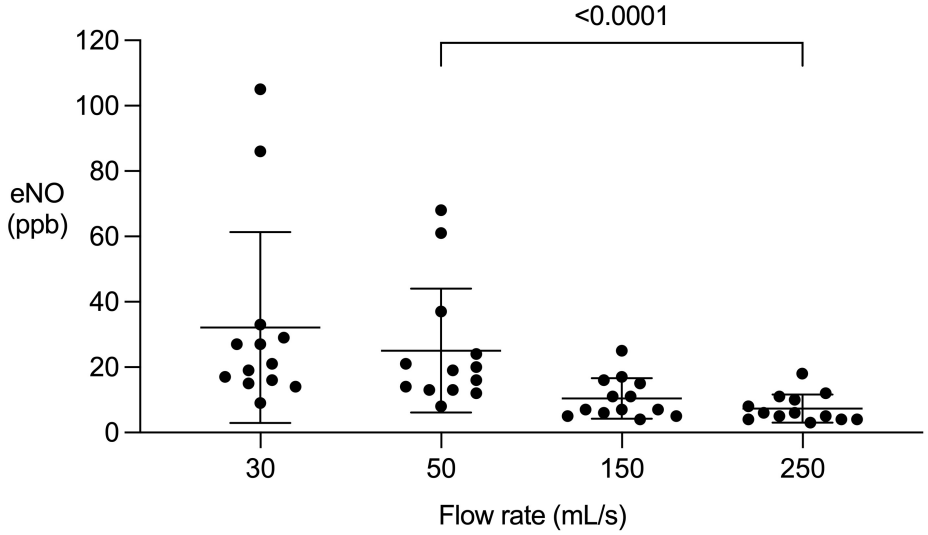


Figure 8. Exhaled nitric oxide (eNO) in 13 healthy controls exhaling at four different flow rates. Friedman test for paired comparison of eNO measured at four expiratory flow rates (30, 50, 150 and 250 mL/s). After inhaling to total lung capacity, the participants exhaled while maintaining a given mouth pressure between 5 and 25 cm $H_2O$ at each flow rate. Participants exhaled in triplicate and the average of the three eNO measurements were plotted for each participant. The data at each flow rate are presented as the mean±SD. ppb: parts per billion

### 4.3 Dietary nitrate effect on exhaled nitric oxide

The control participants (n=20) ingested different volumes of beetroot juice (35, 70 and 140 mL) to determine any possible dose-response between the amount of nitrate-intake and exhaled nitric oxide (Figure 9). Exhaled nitric oxide concentrations did not differ between the different volumes of beetroot juice which are recorded as mean±SD exhaled nitric oxide concentrations of 31±16 ppb, 34±31 ppb and 38±30 ppb (p=0.42, Figure 9) for 35, 70 and 140 mL of beetroot juice, respectively.

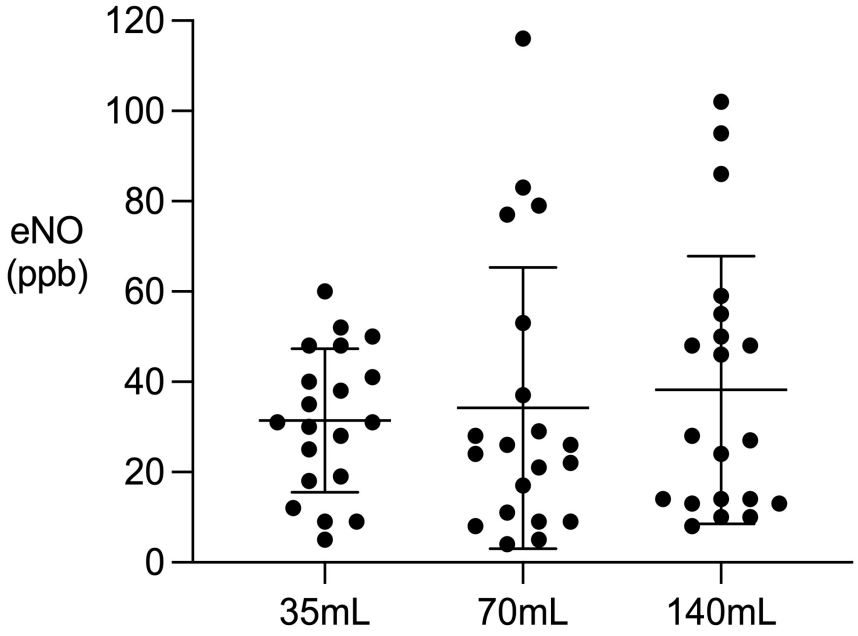


Figure 9. Dose-response of exhaled nitric oxide (eNO) after different volumes of beetroot juice. Friedman test for comparison of eNO from control participants (n=20) at different volumes of beetroot juice (35, 70 and 140 mL). The data at each volume are presented as the mean±SD. ppb: parts per billion

To establish the pattern of nitric oxide production after the consumption of dietary nitrate, members of the physiology department (n=4; all females) were tasked to measure exhaled nitric oxide concentrations over a period of 24 hours after ingesting 70 mL of beetroot juice. After the ingestion of beetroot juice, the average exhaled nitric oxide among the four controls reached peak concentration at 30 minutes post-ingestion (20±8 ppb) after which the concentrations decreased to concentrations comparable to baseline exhaled nitric oxide (Figure 10). A comparison between the exhaled nitric oxide concentrations measured after drinking beetroot juice and exhaled nitric oxide concentrations before beetroot juice ingestion, reported significant differences between two measurement taken in the 24 hours (7±2 ppb, 30 minutes before ingestion vs 20±8 ppb, after beetroot juice; p=0.03). The remainder of the exhaled nitric oxide concentrations measured following 30 minutes post-ingestion did not significantly differ from the exhaled nitric oxide concentrations measured before the participants ingested beetroot juice (Figure 10).

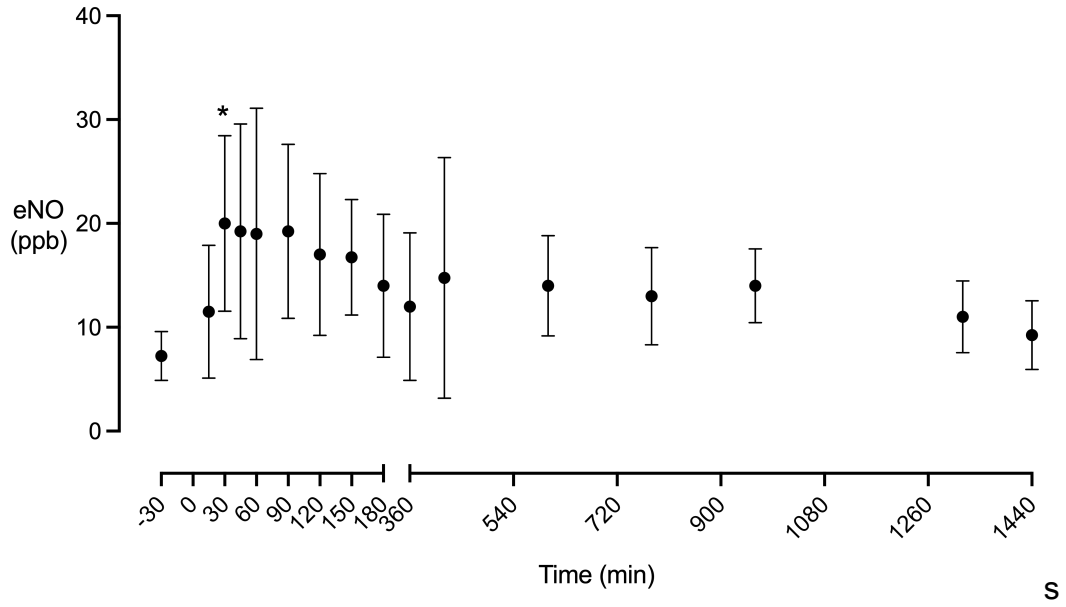


Figure 10. Exhaled nitric oxide (eNO) measured over a period of 24 hours in control participants (n=4) after the ingestion of 70 mL of beetroot juice at time zero minutes. Friedman test performed to compare eNO measured after beetroot juice ingestion to eNO without beetroot juice ingestion. Data are presented as mean±SD. *p=0.03 (Friedman test). ppb: parts per billion

## 4.4 Modulation of oral bacteria

To determine the effect of bacterial flora, in the oral cavity, on exhaled nitric oxide after nitrate-intake, control participants (n=20) rinsed their mouth with antibacterial mouthwash prior to the ingestion of 70 mL beetroot juice. Figure 11 illustrates a comparison between the control participants ingesting beetroot juice without prior mouthwash rinse and the control participants rinsing the mouth with antibacterial mouthwash prior to beetroot ingestion. The mean±SD exhaled nitric oxide concentration in the participants after beetroot juice ingestion was 34±31 ppb, which was significantly higher than when participants rinsed with mouthwash prior to beetroot juice ingestion (18±15 ppb; p=0.02, Figure 11).

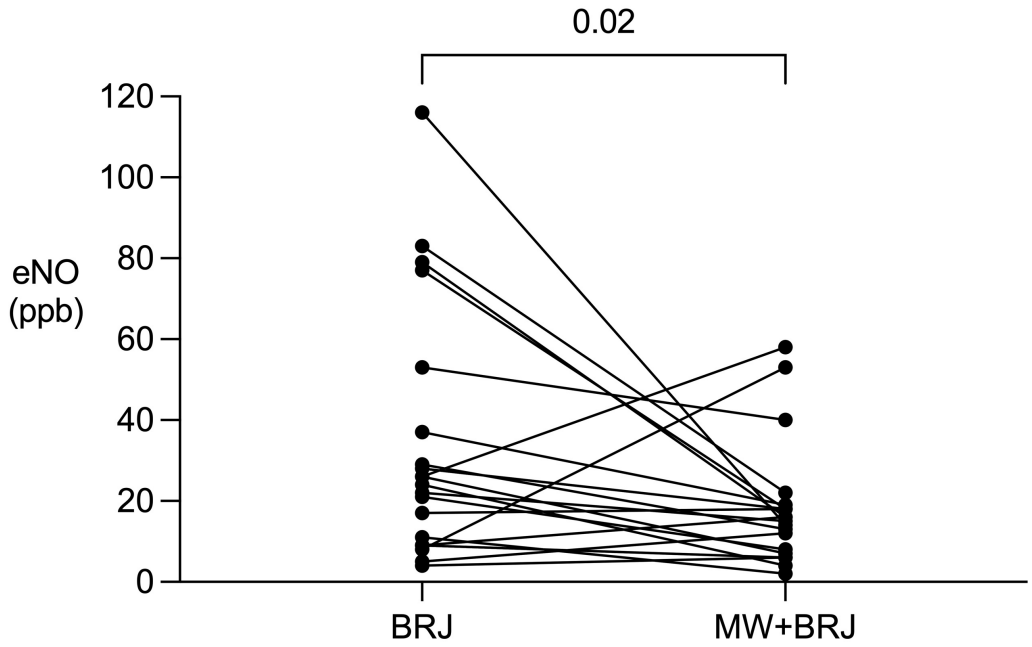


Figure 11. Oral bacteria modulation in control participants (n=20). Wilcoxon test was carried out for the comparison between exhaled nitric oxide (eNO) after beetroot juice ingestion in participants with no prior mouthwash rinse (BRJ) and in participants who rinsed with mouthwash prior to beetroot juice ingestion (MW+BRJ). The mean±SD eNO of the BRJ group was 34±31 ppb compared to 18±15 ppb recorded in the MW+BRJ group. ppb: parts per billion

### 4.5 Modulation of stomach pH

To study the effect of stomach pH on eNO levels, 20 control participants ingested two antacid tablets 30 minutes prior to 70 mL beetroot juice ingestion. Figure 12 illustrates the changes in exhaled nitric oxide concentrations throughout the stomach pH modulation experiment. The baseline exhaled nitric oxide concentrations of each participant were measured every ten minutes for 30 minutes prior to the administration of the antacid tablets. The baseline exhaled nitric oxide concentrations did not differ between the three time-intervals (11±13 ppb, 10±12 ppb and 10±11 ppb; $p>0.05$). The exhaled nitric oxide concentrations at the three time-intervals after antacid administration were also comparable to baseline exhaled nitric oxide (9±11 ppb, 10±12 ppb and 10±13 ppb; $p>0.05$). The eNO levels measured at 5 and 10 minutes after the ingestion of beetroot juice were not significantly different from the eNO levels measured before the beetroot ingestion (14±12 ppb and 13±13 ppb, respectively; Figure 12). Thereafter, the exhaled nitric oxide concentrations significantly increased from 15 to 30 minutes post-beetroot ingestion compared to baseline exhaled nitric oxide (21±22 ppb; $p=0.01$ at 15 minutes post-beetroot ingestion, 24±23 ppb; 21±17 ppb, and 20±14 ppb at 20-, 25- and 30-minutes post-beetroot ingestion, respectfully vs 11±13 ppb; $p<0.0001$, Figure 12).

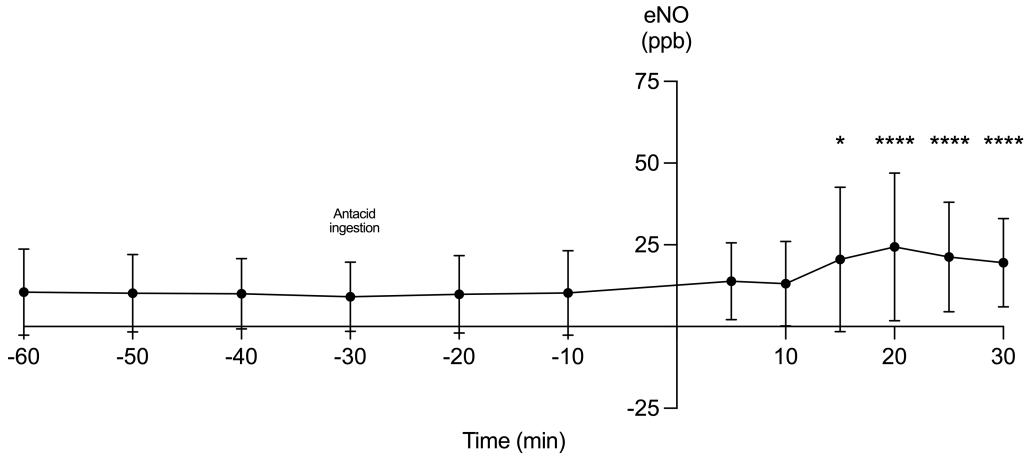


Figure 12. Stomach pH modulation in 20 control participants. Time-series of exhaled nitric oxide (eNO) from the control group who measured eNO before and after antacid ingestion as well as after beetroot ingestion at time zero. Participants consumed two antacid tablets 30 minutes prior to beetroot juice ingestion. Data are expressed as mean±SD. *p=0.01, ****p<0.0001 (Friedman test). ppb: parts per billion

A one-way ANOVA with multiple comparisons was used to determine differences between baseline exhaled nitric oxide concentrations from the 20 control participants and exhaled nitric oxide after beetroot ingestion (9±9 ppb vs 34±31ppb; p= 0.001, Figure 13). Another comparison was made between baseline exhaled nitric oxide and exhaled nitric oxide after antacid use, which found no difference in the concentrations (9±9 ppb vs 10±13 ppb; p>0.99, Figure 13). A comparison between exhaled nitric oxide concentrations after beetroot ingestion and exhaled nitric oxide concentrations after taking antacids followed by beetroot juice consumption was also made, which reported no difference between the two experimental groups (34±31 ppb vs 49±80 ppb; p>0.99, Figure 13). The results indicate no overall decrease in exhaled nitric oxide

after taking antacid tablets prior to 70 mL beetroot juice ingestion and because of this the null hypothesis is therefore rejected.

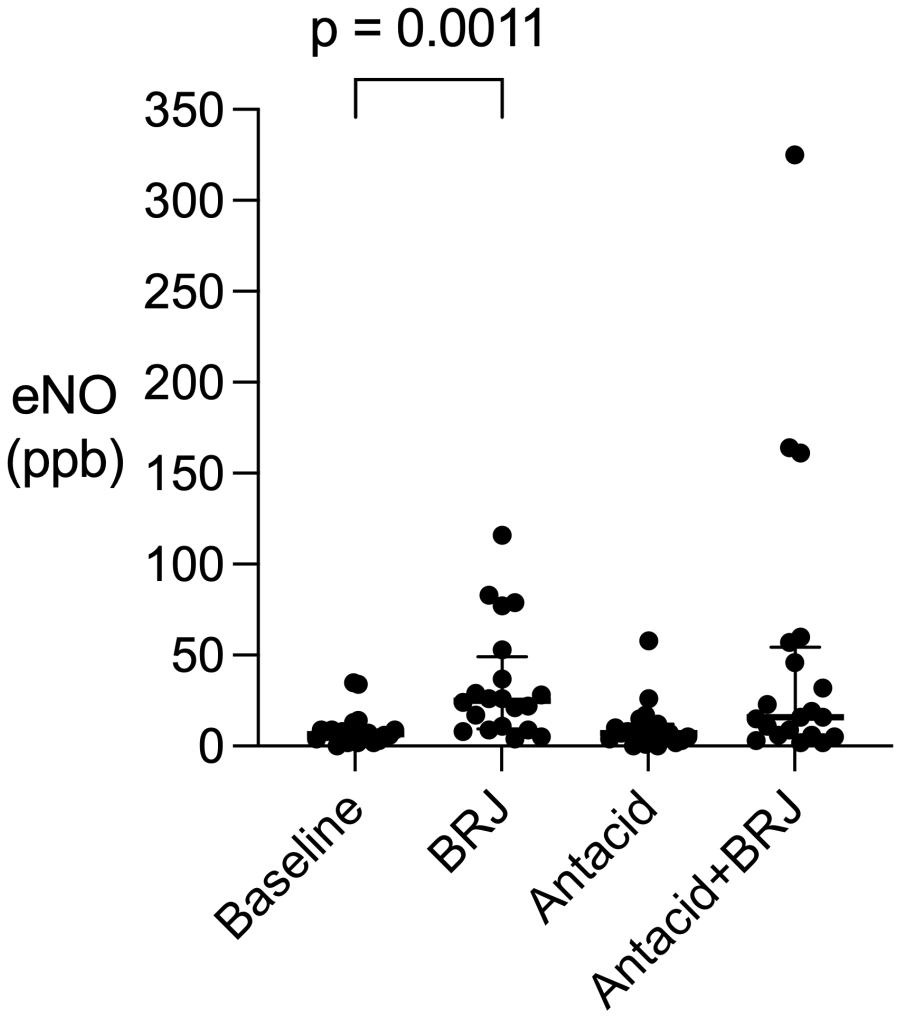


Figure 13. Comparison of antacid and beetroot juice (BRJ) effects on exhaled nitric oxide. One-way ANOVA was used to compare baseline exhaled nitric oxide (eNO) from control participants (n=20) to eNO concentrations after 70 mL beetroot juice ingestion (BRJ) without antacid use as well as a comparison between control participants after taking two antacid tablets prior to beetroot juice ingestion (Antacid) to eNO after antacid use and beetroot juice ingestion (Antacid+BRJ). Two antacid tablets were ingested 30 minutes prior to beetroot juice ingestion. Data are expressed with the median and interquartile range. ppb: parts per billion

# Chapter 5

# 5 Discussion

## 5.1 Endogenous production of exhaled nitric oxide

The primary objective of this quantitative, experimental study was to observe the enzyme-independent pathway of nitric oxide production, which was measured in the airways after ingestion of inorganic dietary nitrate.

Firstly, we demonstrated that exhaled nitric oxide can be measured in healthy participants as well as participants with asthma and those with rhinitis. We made a general finding that asthmatic participants have higher exhaled nitric oxide levels compared to controls and participants with rhinitis. This finding is similar to previous studies that also found significantly higher levels of exhaled nitric oxide in asthmatic subjects due to airway inflammation that is prominent in these subjects (4, 5, 105). Although the average exhaled nitric oxide concentrations of participants with rhinitis in this study were higher than that of control participants, there was no significant difference noted from the analysis ($p=0.21$). This result does not compare to those from previous studies that showed significantly higher levels of exhaled nitric oxide concentrations in participants with rhinitis compared to healthy controls (101, 105). The contrast in results from the rhinitis participants in this study may be due to the smaller sample ($n=7$) measured compared to larger samples in the previous studies (101, 105).

Additionally, this study did not differentiate between participants with atopic and those with non-atopic rhinitis, which may be indicated by the large variation in the exhaled nitric oxide concentrations measured in rhinitis participants (30±31 ppb; coefficient of variation =104%). According to Gratziou et al. (105) patients with atopic rhinitis recorded significantly higher exhaled nitric oxide levels compared to patients with non-atopic rhinitis. Their study also reported comparable exhaled nitric oxide levels

between non-atopic rhinitis patients and healthy controls, which further emphasised the impact of atopy on exhaled nitric oxide levels (105). The findings in their study corresponded with other studies (101, 138) suggesting that the severity of inflammation augments exhaled nitric oxide production in the lower airways.

Similarly, the overlap in exhaled nitric oxide concentrations observed in some participants from the three study groups (i.e., asthmatics, rhinitis, and controls) could also be explained by the stable nature of disease reported in the asthmatic participants, as none of the asthmatics had reported having any exacerbations of symptoms within at least six months prior to the exhaled nitric oxide measurements. Accordingly, studies have shown that exhaled nitric oxide levels measured in controlled asthmatics are comparable to those in healthy controls (5, 138). Scott et al. (138) demonstrated an association between atopy and high levels of exhaled nitric oxide and also found no differences in the average exhaled nitric oxide levels between non-atopic asthmatics compared to non-atopic healthy controls.

Further, the normal lung function studies in addition to the lack of self-reported exacerbations by the participants with asthma suggest very mild and/or well controlled disease (139).

### 5.2 Nitric oxide from the lower respiratory compartments (comparing nitric oxide sources in the airways)

This study has shown that the measurement of exhaled nitric oxide is dependent on the expiratory flow rate, where the higher the flow rate the lower the exhaled nitric oxide concentration compared to the standard flow rate of 50 mL/s (17). The results from this study are comparable to previous studies (2, 23, 140-142) that noted a trend of nitric oxide concentrations to decrease as the expiratory flow rate increases. The possible explanation for this result is that nitric oxide production in the airways occurs

mainly in the proximal/bronchial area, which is represented by lower flow rates while the distal/alveolar area (i.e., represented by higher flow rates) has low nitric oxide levels due to dilution from "nitric oxide-free alveolar gas" (23, 83).

The results in this study only found a significant difference between the highest flow rate (i.e., 250 mL/s) compared to the standard flow rate of 50 mL/s ($p<0.0001$), while the exhaled nitric oxide concentrations from the lower flow rates (i.e., 30 and 50 mL/s) were considerably variable. This high variation at the low flow rates was also noted by Kissoon et al. (140) who concluded that a distinction of inflammation in the airways could be made at low flow rates due to an enhanced nitric oxide signal at those flow rates. This inference could explain the high variation of exhaled nitric oxide among the control participants at low flow rates (i.e., 30 and 50 mL/s) in our study. Two out of 13 controls in our study recorded higher exhaled nitric oxide levels at 30 and 50 mL/s, which may suggest the presence of inflammation in the airway of those two control participants.

### 5.3 The effect of beetroot juice on nitric oxide levels (24-hour pattern and dose-response)

Our study demonstrated that exhaled nitric oxide transiently increased after ingestion of dietary nitrate. Nitric oxide levels are known to be affected by components in the diet that contain nitrate (15, 20, 22, 143). Green, leafy vegetables (e.g., lettuce, spinach and cabbage) as well as root vegetables (e.g., beetroot, radish and fennel) have been shown to have high nitrate content (8). Research has shown that dietary nitrate enters the entero-salivary circulation which concentrates and secretes nitrate in oral saliva (15). Duncan et al. (15) deduced that the amount of nitric oxide formed in the oral cavity is dependent on the amount of nitrite formed from the reduction of dietary

nitrate, which is then further reduced to nitric oxide by acidic pH formed by acid-producing bacteria in the gingiva.

Although the participants in our study demonstrated an increase in exhaled nitric oxide 30 minutes after drinking beetroot juice, the nitric oxide concentrations lowered back down to levels similar to baseline values (i.e., before ingestion) within an hour post-ingestion and remained at levels similar to baseline for the remainder of the 24 hours. This finding is different from results in previous studies that illustrated a sustained increase in exhaled nitric oxide over longer periods of time (20, 22). The reason for the differing results could be due to the small sample (n=4) we measured in our study compared to the larger samples measured in previous studies (20, 22). Another explanation for the transient increase in exhaled nitric oxide might be due to the lower nitrate content (400 mg nitrate) that our participants ingested compared to the higher nitrate content ingestion in previous studies (22, 116). We can speculate further that the low-nitrate content ingested is reduced to nitrite and then to nitric oxide in the entero-salivary pathway. The nitric oxide formed was then sequestered by red blood cells in the circulation (50), which may account for the transient increase of exhaled nitric oxide after nitrate ingestion.

High variation in exhaled nitric oxide was also noted among the participants in our study, specifically after ingestion of the beetroot juice. This large inter-individual variation in exhaled nitric oxide after ingestion along with the small sample size for the 24-hour nitric oxide experiment could also account for the lack of any significant change in exhaled nitric oxide concentrations after dietary nitrate ingestion. This large inter-individual variation has been observed in previous studies as well (30). Exhaled nitric oxide levels are also affected by oral care, whereby increased oral care was found to reduce the amount of exhaled nitric oxide (132). This finding may explain the large

variation in exhaled nitric oxide produced after nitrate ingestion as we did not account for the oral care routine (i.e., despite instructing against use of mouthwash prior to experiments) among the participants in our study before and after ingestion.

In addition to observing the 24-hour pattern of exhaled nitric oxide in healthy participants after nitrate ingestion, our study also aimed to assess whether there was a dose-response effect on exhaled nitric oxide after ingesting different amounts of nitrates (i.e., different volumes of beetroot juice). We found no dose-response relationship using different volumes of beetroot juice but rather observed a large variation in exhaled nitric oxide as the dose increased. This variation could again be explained by the differences in oral care among the participants which affects the amount of exhaled nitric oxide produced via the entero-salivary pathway (132).

### 5.4 Modulation of nitric oxide production (mouthwash experiment and antacid experiment)

With numerous factors affecting nitric oxide production, in two separate experiments we studied the impact of mouthwash and antacid. We found a significant reduction in exhaled nitric oxide when antibacterial mouthwash was used prior to nitrate ingestion. This aligns with a previous study that looked at the effects of mouthwash on nitric oxide (30). Zetterquist et al. (30) used a mouthwash with a similar composition to the mouthwash we used in our study (chlorhexidine acetate) and found an immediate attenuation of nitric oxide concentrations, especially when participants ingested a nitrate load prior to the mouthwash use. Our study also showed an immediate reduction in exhaled nitric oxide, even though the participants in our study rinsed the mouth with mouthwash prior to nitrate ingestion. Alternatively, Govoni et al. (62) determined the effect that mouthwash had on plasma and salivary nitrate and nitrite after ingestion of dietary nitrate. Although the study did not measure exhaled nitric oxide, they reported

an acute increase in plasma nitrite post-nitrate ingestion. They found that antibacterial mouthwash inhibited nitrate reductase activity in the oral cavity, therefore abolishing the formation of nitrite in saliva as well as significantly reducing plasma nitrite (62).

The reduction in exhaled nitric oxide after mouthwash use is explained by the bactericidal properties of the mouthwash that reduces plaque and gingivitis (144). The reduction in these oral bacteria would then attenuate the reduction of oral nitrate to nitrite, subsequently reducing the formation of nitric oxide (132).

Furthermore, we also modulated the stomach pH of healthy participants using antacid tablets and observed a non-significant transient reduction in exhaled nitric oxide after nitrate ingestion (i.e., with prior antacid treatment). Our findings agree with the results from a similar study that showed no significant change in exhaled nitric oxide when the participants were pretreated with a proton pump inhibitor (29). Although our findings agree with a previous study (29) it may also be that we did not administer a sufficient dose of antacid to illicit a significant change in the stomach pH.

Lundberg et al. (29) also measured nitric oxide from gas expelled from the stomach of healthy and asthmatic participants where they found the stomach to be a rich source of nitric oxide formation from reduction of nitrite catalysed by the acidic environment. They also confirmed the dependency of nitric oxide formation on the stomach pH when they noted a significant attenuation of nitric oxide in gas expelled from the stomach after pretreatment with a selective proton pump inhibitor (29). The finding in our study further confirms that even though the stomach is a source of high nitric oxide levels (29), it does not contribute to the exhaled nitric oxide levels as observed by Lundberg et al. (29).

# Chapter 6

# 6 Conclusions and recommendations

## 6.1 Conclusions

Altogether, exhaled nitric oxide can be measured in asthmatics, rhinitis and healthy participants. The asthmatic participants exhibited higher exhaled nitric oxide compared to the healthy and rhinitis participants. The overlap in exhaled nitric oxide concentrations between the three study groups may be attributed to atopy, which was not controlled for in the participants of this study. Exhaled nitric oxide is also dependent on the expiratory flow rate, which has an inverse effect on exhaled nitric oxide. This finding indicates that flow rate can be used to distinguish between exhaled nitric oxide levels sourced from the distal (i.e., bronchial) and the proximal (i.e., alveolar) airways (23, 83). This study also found a transient increase in exhaled nitric oxide from dietary nitrate in healthy participants. We also noted large variation after dietary nitrate was ingested by healthy participants. The large variation could explain the inconsistencies in findings from previous studies around the impact that nitrate supplementation has on the cardiovascular system, especially related to exercise performance (37, 38, 55).

Lastly, with the impact that antibacterial mouthwash has on the production of nitric oxide, we speculate that oral care and mouthwash use should be considered when individuals supplement with dietary nitrate as the bactericidal effects of the mouthwash abolish the conversion of nitrate to nitrite. Our findings also confirm that even though the stomach is structurally connected to the oral cavity via the oesophagus, the abundant production of nitric oxide from the stomach does not influence exhaled nitric oxide. This may suggest the local conversion of nitrate to nitrite and subsequently to nitric oxide in the oral cavity, as stomach pH modulation did not impact the increase in exhaled nitric oxide after nitrate ingestion.

## 6.2 Recommendations

Although some of our study findings agree with results in literature, there are a few aspects that should still be addressed to clarify our findings. Firstly, the small sample size in this research study reduced the power of our findings, especially for the asthmatic and rhinitis participant groups. A larger sample size may also reduce the amount of variation noted after beetroot juice ingestion in healthy participants and increase the power of the study. Therefore, further research is required, using a larger sample size comparing exhaled nitric oxide in asthma and rhinitis compared to healthy controls. Secondly, considering the overlap in exhaled nitric oxide levels among the different study groups, future research should consider the atopic status of each participant, and its effects on baseline exhaled nitric oxide. As rhinitis includes inflammation of the nasal cavity, a more discernible distinction between asthmatics and participants with rhinitis could be made by measuring exhaled nitric oxide from the nasal cavity specifically. Lastly, our study did not measure the stomach pH of the participants after antacid administration. Further investigations should consider the measurement of stomach pH before and after the administration of antacid in order to ensure a change occurred in the stomach pH and its effect on nitric oxide formation. These recommendations would strengthen and clarify the conclusions described above.

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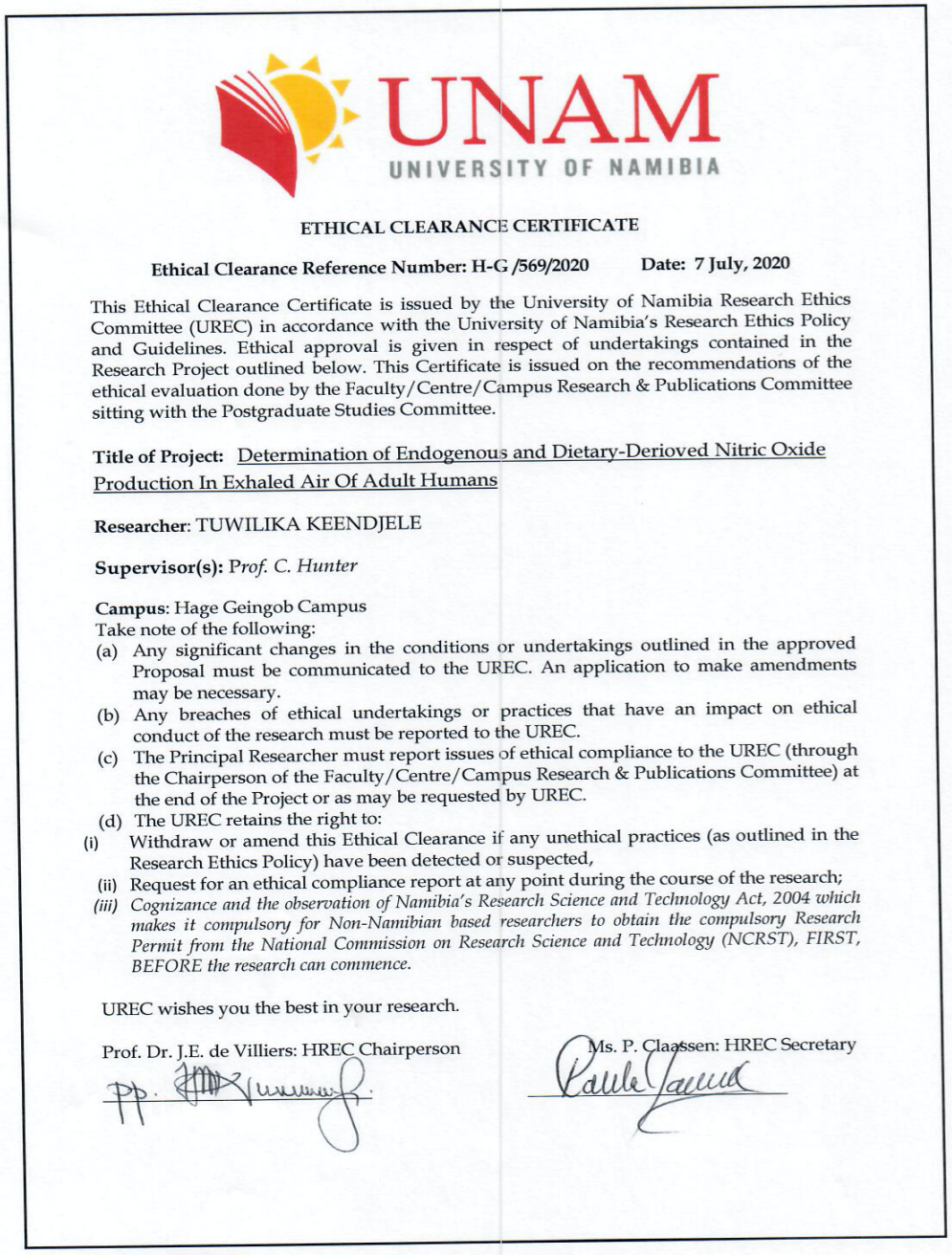
# Appendices

A. Appendix 1: Ethical clearance certificate



UNAM
UNIVERSITY OF NAMIBIA

**ETHICAL CLEARANCE CERTIFICATE**

**Ethical Clearance Reference Number: H-G /569/2020** **Date: 7 July, 2020**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

**Title of Project:** Determination of Endogenous and Dietary-Derioved Nitric Oxide Production In Exhaled Air Of Adult Humans

**Researcher**: TUWILIKA KEENDJELE

**Supervisor(s):** *Prof. C. Hunter*

**Campus**: Hage Geingob Campus

Take note of the following:

(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.

(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.

(c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.

(d) The UREC retains the right to:

(i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

(ii) Request for an ethical compliance report at any point during the course of the research;

*(iii) Cognizance and the observation of Namibia's Research Science and Technology Act, 2004 which makes it compulsory for Non-Namibian based researchers to obtain the compulsory Research Permit from the National Commission on Research Science and Technology (NCRST), FIRST, BEFORE the research can commence.*

UREC wishes you the best in your research.

Prof. Dr. J.E. de Villiers: HREC Chairperson

Ms. P. Claassen: HREC Secretary

B. Appendix 2: Ministry of Health and Social Services research approval

**REPUBLIC OF NAMIBIA**

*Ministry of Health and Social Services*


Private Bag 13198
Windhoek
Namibia

Ministerial Building
Harvey Street
Windhoek

Tel: 061 – 203 2537
Fax: 061 – 222558
E-mail: itashipu87@gmail.com


**OFFICE OF THE EXECUTIVE DIRECTOR**

**Ref:** 17/3/3/TK
**Enquiries:** Mr. A. Shipanga

**Date:** 03 December 2020

**Ms. Tuwilika Keendjele**
**Private Bag 13301**
**Windhoek**
**Namibia**

Dear Ms. Keendjele

***Re:* Determination of endogenous and dietary-derived nitric oxide production in exhaled air of adult humans.**

1. Reference is made to your application to conduct the above-mentioned study.
2. The proposal has been evaluated and found to have merit.
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**

3.1 The data to be collected must only be used for academic purpose;
3.2 No other data should be collected other than the data stated in the proposal;
3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;
3.4 A quarterly report to be submitted to the Ministry's Research Unit;
3.5 Preliminary findings to be submitted upon completion of the study;
3.6 Final report to be submitted upon completion of the study;
3.7 Separate permission should be sought from the Ministry for the publication of the findings.

4. All the cost implications that will result from this study will be the responsibility of the applicant and **not** of the MoHSS.

EXECUTIVE DIRECTOR
MINISTRY OF HEALTH AND SOCIAL SERVICES

Yours sincerely,

**BEN NANGOMBE**
**EXECUTIVE DIRECTOR**

*"Your Health Our Concern"* 10/14843

C. Appendix 3: Participant information leaflet and consent form

# PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

**TITLE OF THE RESEARCH PROJECT:** Determination of endogenous and dietary-derived nitric oxide production in exhaled air of adult humans

**REFERENCE NUMBER:** H-G /569/2020

**PRINCIPAL INVESTIGATOR:** Tuwilika PT Keendjele

**ADDRESS:** tkeendjele@unam.na

**CONTACT NUMBER:** 0812421075

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Research Ethics Committee at The University of Namibia and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and Namibian National Research Ethics Guidelines.

## 1. What is this research study all about?

a) *The research will be conducted in the Physiology Postgraduate laboratory at the School of Medicine, Hage Geingob Campus, University of Namibia. We aim to recruit a total of 70 participants from the Hage Geingob Campus and surrounding areas (if need be).*

b) *Nitrates can be found in food products such as green, leafy vegetables (e.g. lettuce, and spinach) and beetroot. These dietary nitrates, especially in the form of beetroot juice, have been studied in individuals with airway diseases (e.g., asthma); cardiovascular problems, such as high blood pressure; and in athletes to determine if there is an improvement in their activity levels. The reason for our research is to measure the breakdown products of nitrate (in beetroot juice) by measuring them exhaled air. We will also look at the pathway of dietary nitrates (i.e., in the form of beetroot juice) in healthy individuals, specifically in their airways.*

c) *Explain all procedures.*

***Anthropometry and spirometry:*** *We will measure your normal physiological parameters, which are your height, weight, blood pressure and heart rate. We will also measure your exhaled nitric oxide levels and test your ling function with a spirometer.*

***Part 1****: We will measure your exhaled nitric oxide levels by asking you to breathe into the nitric oxide analyser three consecutive times. We will demonstrate how to inhale and then exhale into the analyser and continue to instruct you as you breath into the analyser. We will ask you to exhale at different flow rates to distinguish between the nose, the upper airway and the lower airway. This part of the experiment will be conducted on* ***all*** *the volunteers (i.e., healthy, asthmatics and those with rhinitis).*

***Part 2****: This part of the experiment will take place on four separate days, with a rest day in-between each experimental day. We will measure your exhaled nitric oxide levels by asking you to breathe into the nitric oxide analyser three times. After that, we will give you one of three volumes, either 35, 70 or 140 mL of beetroot juice. On each separate experimental day, you will be given another dose, until you have ingested all three different doses.*

***Part 3****: This experiment will take place over a period of 24 hours. We will measure your exhaled nitric oxide levels by asking you to breathe into an analyser three times. We will then ask to drink beetroot juice (70 mL). After ingestion, we will ask you to record your exhaled nitric oxide levels, using a portable analyser that you will take home. We will provide you with a data sheet that you can fill out as you measure your exhaled nitric oxide.*

***Part 4****: We will measure your exhaled nitric oxide levels by asking you to breathe into the nitric oxide analyser three times. We will then ask you to rinse your mouth with 20 mL of antibacterial mouthwash (Corsodyl) for 60 seconds. After that we will ask you to rinse your mouth with 30 mL of beetroot juice. We will then measure your exhaled breath every five minutes for a period of 30 minutes.*

***Part 5****: During this experiment we will first measure your exhaled air at three-time intervals that will be 10 minutes apart. After that we will ask you to take two Rennie® chewable tablets. We will then measure your exhaled air at three times intervals that are 10 minutes apart. We will then ask you to drink beetroot juice and we will continue to measure your exhaled nitric oxide every 5 minutes over a period of 30 minutes.*

*d) Explain any randomisation process that may occur.*

***All** participants (healthy, asthmatic and those with rhinitis) will take part in **Anthropometry and Spirometry** as well as **Part 1** of the project. Only the **healthy** participants will take part in **Parts 2, 3, 4** and **5** of the project.*

*e) Explain the use of any medication, if applicable.*

*Corsodyl is a commercially available anti-bacterial mouthwash that can be found in supermarkets. You will be asked to rinse out your mouth with this mouthwash as part of one of the experiments in this research project.*

*Rennie® Peppermint Chewable tablets are antacid used to treat heartburn and gastric hyperacidity. Each tablet contains calcium carbonate (680 mg) and magnesium carbonate (80 mg) as their active ingredients. The recommended dosage is usually 1-2 tablets for adults and children over the age of 12 years. These tablets are commercially available as over-the-counter medication found both in pharmacies and supermarkets. You will be asked to take the maximum recommended dose of two tablets for one of the experiments in this research project.*

## 2. Why have you been invited to participate?

*a) You have been invited to participate in this study either because you are a healthy individual or an individual diagnosed with asthma or currently experiencing rhinitis (i.e., inflamed and runny nasal passages). Our study will be measuring exhaled nitric oxide all three types of individuals stated. Individuals with asthma or with rhinitis will be compared to healthy individuals in terms of their exhaled nitric oxide measurements that are known to be higher than those in healthy individuals.*

## 3. What will your responsibilities be?

*a) Your responsibility as a participant will be to avoid nitrate-rich foods, which will be listed for you before the beginning of each experiment. Those of you participating in the 24-hour experiment will also be required to diligently take your measurements while at home and to take proper care of the equipment while it is in your possession. You will also be responsible for returning said equipment to the research lab immediately after the 24-hour experiment. Your responsibility is to report any concerns or discomfort experienced during the experiments. Lastly, the participant will have the responsibility of informing us, researchers in the study, should you no longer wish to participate.*

*b) See attached Informed Consent Form.*

## 4. Will you benefit from taking part in this research?

**a)** This study may not have a direct benefit to you, but the results from this study may increase the knowledge around the physiological role of dietary nitrate (beetroot juice) in **healthy individuals** only. The ability to measure exhaled nitric oxide in the airways, may inform local clinicians to consider the use of this method as a diagnostic tool for patients with asthma and other inflammatory airway conditions.

## 5. Are there in risks involved in your taking part in this research?

Inorganic nitrate (beetroot juice; Beet It (Heartbeet Ltd)) that will be consumed by the participants, is a commercially available sports drink and dietary supplement. The beetroot juice that will be ingested is a natural product containing concentrated beetroot juice (98%) and lemon juice (2%). Each shot of Beetit Sport contains a minimum of 0.4 to 0.45 g of dietary nitrate. The main ingredient in beetroot juice is inorganic nitrate ($NO_3^-$) which is natural occurring product found in vegetables (e.g., beetroot, spinach, lettuce, etc.) as well as water. Nitrates are also used as preservatives and additives in food, especially in meat products (Santamaria, 2006). You will be given the maximum tolerated dose of beetroot juice.

Chlorhexidine (0.2%) (Corsodyl, GlaxoSmithKline Consumer Healthcare, South Africa (Pty) Ltd) is a commercially available anti-bacterial mouthwash. This mouthwash is recommended for daily use to maintain oral hygiene. You will be asked to rinse your mouth with the mouthwash.

Rennie® (Peppermint Chewable tablets, Bayer (Pty) Ltd.) are antacids used to treat heartburn and gastric hyperacidity. Each tablet contains calcium carbonate (680 mg) and magnesium carbonate (80 mg) as their active ingredients. The recommended dosage is usually 1-2 tablets for adults and children over the age of 12 years. These tablets are commercially available as over-the-counter medication found both in pharmacies and supermarkets. You will be asked to take the maximum recommended dose of Rennie®.

Side effects of Rennie® include rare allergic effects such as rash, itching, difficulties breathing, swelling face, mouth or throat and anaphylactic shock Severe-life threatening allergic reaction may cause low blood pressure, shock, heart palpitations, difficulties breathing, bronchospasm, skin reactions, abdominal pains or cramps, vomiting and diarrhoea. Side effects are unlikely to occur at the recommended dosages.

## 6. If you do not agree to take part, what alternatives do you have?

*b) Not applicable. Participants who do not wish to take part may refuse with no consequences.*

## 7. Who will have access to your medical records? (Where applicable)

*a) Not applicable.*

## 8. What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

*a) Clarify issues related to insurance cover if applicable. If any pharmaceutical agents are involved will compensation be according to ABPI guidelines? (Association of British Pharmaceutical Industry compensation guidelines for research related injury which is regarded as the international gold standard). If yes, please include the details here. If no, then explain what compensation will be available and under what conditions.*

## 9. Will you be paid to take part in this study and are there any costs involved?

*You will be given a sandwich and a cool drink after each visit to the laboratory.*

## *10.* Is there anything else that you should know or do?

*a) You should inform your family practitioner or usual doctor that you are taking part in a research study. (Include if applicable)*

b) *You should also inform your medical insurance company that you are participating in a research study. (Include if applicable)*

c) *You can contact Prof Hunter at tel +264 818679668 if you have any further queries or encounter any problems.*

d) *You can contact the Centre for Research and Publications at* **+264 061 2063061;** **pclaassen@unam.na** *if you have any concerns or complaints that have not been adequately addressed by the investigator.*

e) *You will receive a copy of this information and consent form for your own records.*

## 11. Declaration by participant

By signing below, I …….. agree to take part in a research study entitled *Determination of endogenous and dietary-derived nitric oxide production in exhaled air of adult humans*.

**I declare that:** I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

a) I have had a chance to ask questions and all my questions have been adequately answered.

b) I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

c) I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

d) I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ……. on (*date*) …….. 2021.

Signature of participant Signature of witness

## 12. Declaration by investigator

I *(name)* declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ..... on (*date*) ……..…… 2021.

Signature of investigator Signature of witness

## 13. Declaration by interpreter

I *(name)*declare that:

a) I assisted the investigator (*name*) …… to explain the information in this document to (*name of participant*) …….. using the language medium of (Oshiwambo, Oshiherero, Afrikaans, etc.)

D. Appendix 4: Data collection sheet

**Research project:** Determination of endogenous and dietary-derived nitric oxide production in exhaled air of adult humans

**Equipment and consumables list**

| **Equipment** | |
|---|---|
| Sievers NOA 280i | |
| Vacuum pump | |
| Analog to digital converter | |
| NOBreath® analyser | |
| NOBreath® charging dock | |
| Ambulatory blood pressure monitor | |
| EasyOne® Air Spirometer | |
| Scale | |
| Stadiometre | |
| **Consumables** | |
| Medial oxygen tank | |
| Zero air filter | |
| Flow restrictors (1, 2, 4, 6) | |
| Disposable viral/bacterial filters | |
| NOBreath® Single-use mouthpiece | |
| Cleansing wipes | |
| Beetit organic beetroot juice shots | |
| Noseclips | |
| Alcohol pads | |
| EasyOne Flow Tube respiratory tubes | |
| Rennie® Peppermint Chewable tablets | |
| Corsodyl mouthwash (Original) | |

Participant number: _____

Cell number: _____

Gender (circle participant's gender): Male Female

Age (DOB): ___/____/_____ (day/month/year)

Weight (kg): _____

Height (cm): _____

BMI (kg/m$^2$): _____

**Inclusion criteria (either circle' Y' for yes or 'N' for no)**

***Healthy participants***

1. Are you a smoker? Y / N

5. Do you have any pain or physical problem that may prevent you from taking a deep breath and exhaling forcefully? Y / N

6. Did you vigorously exercise in the last hour? Y / N

7. Did you have anything to eat or drink in the past hour? Y / N

8. Have you eaten beetroot, radishes, broccoli or green leafy vegetables (i.e., celery, spinach, lettuce, cabbage) in the last 12 hours? Y / N

9. Have you eaten processed meats such as bacon, ham, salami, other cold meats, or smoked fish in the past 12 hours? Y / N

10. Have you had a cough, cold, phlegm, runny nose or other respiratory illness in the past week? Y / N

11. Have you been diagnosed with any chronic disease? Y / N

12. Is there a possibility of you being pregnant? Y / N

13. Are you willing to take a pregnancy test? Y / N

Met all inclusion criteria Y / N

Met all exclusion criteria Y / N

***Asthmatic participant***

Do you have a history of wheezing? Y / N

Do you have a history of tightness in your chest? Y / N

Have you been diagnosed with asthma by your doctor? Y / N

Are you taking any medication for the asthma (i.e., inhaled Beta2-agonists and/or inhaled corticosteroids) Y / N?

If yes to question 4, specify which medication you are using, if no skip to question 6

_____

14. Do you have any pain or physical problem that may prevent you from taking a deep breath and exhaling forcefully? Y / N

15. Did you vigorously exercise in the last hour? Y / N

16. Did you have anything to eat or drink in the past hour? Y / N

17. Have you eaten beetroot, radishes, broccoli or green leafy vegetables (i.e., celery, spinach, lettuce, cabbage) in the last 12 hours? Y / N

18. Have you eaten processed meats such as bacon, ham, salami, other cold meats, or smoked fish in the past 12 hours? Y / N

Met all inclusion criteria Y / N

Met all exclusion criteria Y / N

***Rhinitis participant***

Do you have a history of seasonal allergies (i.e., nasal itching, sneezing, runny nose, difficulty breathing, itching, redness or tearing of the eyes)? Y / N

Do you take any medication for the rhinitis? Y / N

If yes to question 2, specify which medication you take, if no skip to question 4.

_____

19. Do you have any pain or physical problem that may prevent you from taking a deep breath and exhaling forcefully? Y / N

20. Did you vigorously exercise in the last hour? Y / N

21. Did you have anything to eat or drink in the past hour? Y / N

22. Have you eaten beetroot, radishes, broccoli or green leafy vegetables (i.e., celery, spinach, lettuce, cabbage) in the last 12 hours? Y / N

23. Have you eaten processed meats such as bacon, ham, salami, other cold meats, or smoked fish in the past 12 hours? Y / N

Met all inclusion criteria Y / N

Met all exclusion criteria Y / N

Date of informed consent: ___/___/_____ (day/month/year)

**General notes:**

During all measurements, ensure that you are seated comfortably and in an upright position.
Avoid consumption of any alcoholic beverage at least 24 hours prior to the experiment.
Avoid caffeinated drinks such as coffee or coca cola.
Maintain your normal diet and avoid the foods listed below:
Rocket, spinach, lettuce, radish, beetroot, cabbage, cold meats (i.e., ham and salami), cured meat (i.e., biltong), smoked fish.
Avoid eating, drinking and exercising one hour before the experiments.

**Exhaled nitric oxide (ppb)**

| **Measurement 1** | **Measurement 2** | **Measurement 3** | **Average** |
|---|---|---|---|
| | | | |

**Blood pressure**

| Parameter | Measurement 1 | Measurement 2 | Measurement 3 | Average |
|---|---|---|---|---|
| Systolic BP (mmHg) | | | | |
| Diastolic BP (mmHg) | | | | |
| Heart rate (beats/min) | | | | |

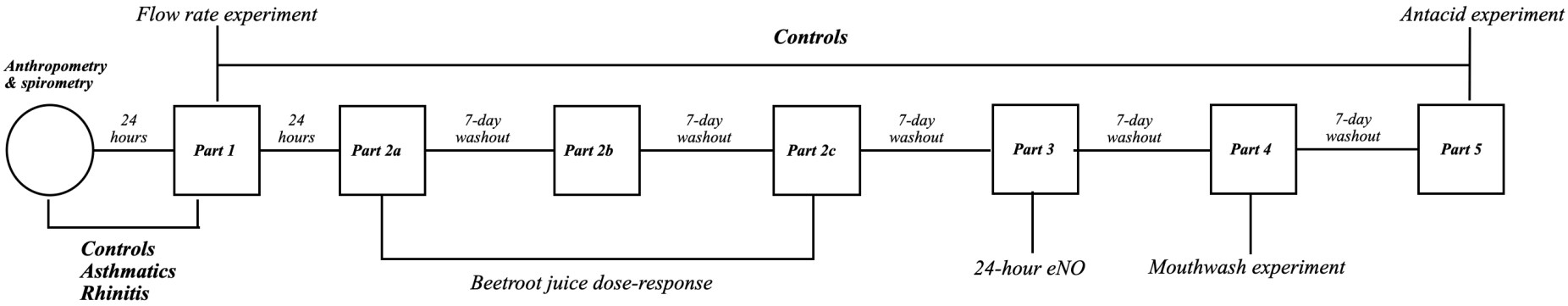
**Spirometry measurements**

| Parameter | Measurement 1 | Measurement 2 | Measurement 3 | Best |
|---|---|---|---|---|
| FEV1 (L) | | | | |
| FVC (L) | | | | |
| FEV1/FVC (%) | | | | |
| PEF (L/min) | | | | |

**Experiment group enrolment (circle category of participant)**

1. Control: Healthy participants who do not have asthma or rhinitis. Control participants; complete **parts**, **one**, **two**, **three**, **four** and **five**.
2. Asthmatics: Healthy participants who were previously diagnosed with asthma. Asthmatic participants; complete baseline physiological measurements (**anthropometry** and **spirometry**) and **part one**.
3. Rhinitis: Participants who are currently experiencing the following symptoms; itching, sneezing, runny nose, stuffiness and itchy and watery eyes. Rhinitis participants; complete baseline physiological measurements (**anthropometry** and **spirometry**) and **part one**.

## Experiment timeline



**EXPERIMENTS**

**PART ONE (FLOW RATE EXPERIMENT: ENO MEASUREMENTS FROM RESPIRATORY COMPARTMENTS)**

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

NO analyser: Sievers 280i

| **Respiratory compartment** | **Flow rate (mL/s)** | **1. eNO (ppb)** | **2. eNO (ppb)** | **3. eNO (ppb)** | **Average eNO (ppb)** |
|---|---|---|---|---|---|
| Tracheal | | | | | |
| | 30 | | | | |
| | 50 | | | | |
| Alveolar | | | | | |
| | 150 | | | | |
| | 250 | | | | |

**PART TWO (24-HOUR ENO MEASUREMENT)**

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

NO analyser: NOBreath®

| **Description** | **Time** | **Time intervals (minutes)** | **1. eNO (ppb)** | **2. eNO (ppb)** | **3. eNO (ppb)** | **Average (ppb)** |
|---|---|---|---|---|---|---|
| Baseline | | -30 | | | | |
| | | -20 | | | | |
| | | -10 | | | | |
| Beetroot juice ingestion | | 0 | | | | |
| | | 15 | | | | |
| | | 30 | | | | |
| | | 45 | | | | |
| | | 60 | | | | |
| | | 90 | | | | |
| | | 120 | | | | |
| | | 150 | | | | |
| | | 180 | | | | |
| | | 240 | | | | |
| | | 300 | | | | |
| | | 360 | | | | |
| | | 420 | | | | |
| | | 600 | | | | |
| | | 780 | | | | |
| | | 960 | | | | |
| | | 1320 | | | | |
| | | 1440 | | | | |

**PART THREE (DOSE-RESPONSE)**

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

NO analyser: NOBreath®

| **Description** | **Time intervals (minutes)** | **Beetroot juice dose (mL)** | **1. eNO (ppb)** | **2. eNO (ppb)** | **3. eNO (ppb)** | **Average (ppb)** |
|---|---|---|---|---|---|---|
| Baseline | -30 | | | | | |
| | -20 | | | | | |
| | -10 | | | | | |
| Beetroot juice ingestion | 0 | **35** | | | | |
| | 10 | | | | | |

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

| **Description** | **Time intervals (minutes)** | **Beetroot juice dose (mL)** | **1. eNO (ppb)** | **2. eNO (ppb)** | **3. eNO (ppb)** | **Average (ppb)** |
|---|---|---|---|---|---|---|
| Baseline | -30 | | | | | |
| | -20 | | | | | |
| | -10 | | | | | |
| Beetroot juice ingestion | 0 | **70** | | | | |
| | 10 | | | | | |

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

| **Description** | **Time intervals (minutes)** | **Beetroot juice dose (mL)** | **1. eNO (ppb)** | **2. eNO (ppb)** | **3. eNO (ppb)** | **Average (ppb)** |
|---|---|---|---|---|---|---|
| Baseline | -30 | | | | | |
| | -20 | | | | | |
| | -10 | | | | | |
| Beetroot juice ingestion | 0 | **140** | | | | |
| | 10 | | | | | |

**PART FOUR (MOUTHWASH EXPERIMENT: ORAL BACTERIA MODULATION)**

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

NO analyser: NOBreath®

| **Description** | **Time** | **Time interval (minutes)** | **eNO (ppb)** |
|---|---|---|---|
| Baseline | | -15 | |
| | | -10 | |
| | | -5 | |
| Mouthwash rinse | | | |
| Beetroot juice ingestion | | 0 | |
| | | 5 | |
| | | 10 | |
| | | 15 | |
| | | 20 | |
| | | 25 | |
| | | 30 | |

**PART FIVE (ANTACID EXPERIMENT: STOMACH PH MODULATION)**

Date of experiment: ___/___/_____(day/month/year)

Time of experiment: _____ (24-hour clock)

NO analyser: NOBreath®

| **Description** | **Time** | **Time interval (minutes)** | **1. eNO (ppb)** | **2. eNO (ppb)** | **3. eNO (ppb)** | **Average (ppb)** |
|---|---|---|---|---|---|---|
| Baseline | | -60 | | | | |
| | | -50 | | | | |
| | | -40 | | | | |
| Ingest two antacid (Rennie®) tablets | | | | | | |
| Measure eNO after antacid ingestion | | | | | | |
| | | -30 | | | | |
| | | -20 | | | | |
| | | -10 | | | | |
| Beetroot juice ingestion | | | | | | |
| | | 0 | | | | |
| | | 5 | | | | |
| | | 10 | | | | |
| | | 15 | | | | |
| | | 20 | | | | |
| | | 25 | | | | |
| | | 30 | | | | |