Nasal Nitric Oxide

Methodology, normal values and potential clinical application

door

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PART 1

INTRODUCTION

CHAPTER 1.1

SCOPE OF THIS THESIS

Nasal nitric oxide (nNO) concentrations are known to be high in the nose and paranasal sinuses. Nasal NO is also known to be influenced by different (upper) airway conditions. Yet, there is no strict consensus on how to measure nNO resulting in contradictions on how (upper) airway diseases influence nNO. Nevertheless, nNO is suggested as a high potential in the role of a diagnostic and/or monitoring tool for upper airway diseases. In this thesis we explore nNO measurement methodology, normal values and its possible use in clinical practice.

Nitric oxide is a gas, highly reactive and first considered only a noxious air pollutant. However, *in vivo* it is believed to play a vital role in many biological events including the regulation of blood flow, platelet function, immunity and neurotransmission. NO is formed by the action of a specific enzyme known as NO synthase (NOS). There are three NOS isoforms, all identified in the human airway mucosa. The neuronal type (nNOS), endothelial-type (eNOS) and the inducible type (iNOS). The first two, nNOS and eNOS, are constitutively expressed and generate relatively low levels of NO. The inducible type, iNOS, on the other hand, is generally expressed only in response to external stimuli, such as certain cytokines and bacterial products.
NO is found in both the upper and lower airways. Though, baseline NO levels generated in the nasal cavities and paranasal sinuses i.e. the nose, are several hundred times higher compared to NO levels generated in the lower airways i.e. the lungs. The origin of nNO is not completely clear. It is proposed that it might be the result of diffusion of NO from the paranasal sinuses into the nasal cavity. Yet, it might also be produced by the nasal epithelium itself. The high nNO levels are suggested to improve ciliary beat frequency and to inhibit the multiplication of bacteria, viruses, fungi and parasites.

Measuring nNO can be done in different ways; it can be extracted passively or actively with different aspiration flows, from one or two nostrils, during breathing quietly or during breathhold. There is not yet a distinct unanimity regarding the measurement methodology. Consequently, there are a lot of conflicting findings regarding the effect of upper airway diseases on nNO. Additively, only recently, one research group described a different nNO measurement procedure. They showed that exhalation while 'humming', that is phonating an 'm' during the measurement, resulted in a quick instant increase in the nNO concentration to a peak value. This peak, they hypothesized, probably gives an indication on the patency of the nasal cavity to the paranasal sinuses (ostiomeatal complex). If this is true, humming during nNO measurements will give additive information regarding the patency of the upper airways. The effect of humming has only been assessed on a very small scale and needs to be further examined.

In **chapter 1.2** of this thesis we will more extensively describe the origin of nNO followed by the different measurement methods. We will go into the effect of allergic rhinitis on nNO, as well as the effect of nasal provocation and nasal medication on

nNO. In the **2nd part** we explore nNO measurements. In **chapter 2.1** we examine the effect of aspiration flow, the nasal cycle and the reproducibility on nNO concentrations in air sampled from one nostril during breathhold. In **chapter 2.2 and 2.3** we report nNO normal values in children and adults. In addition we look into the effect of humming on nNO concentrations in adult subjects. The **third part** comprises the exploration of nNO concentrations in patients with allergic rhinitis, nasal polyps (**chapter 3.1**) and in patients with cystic fibrosis (**chapter 3.2**). Finally, **part 4** concludes this work by a discussion on the methodological strategies, the results and the possible implications of our findings with regards to the question whether nNO has a potential role in clinical practice.

CHAPTER 1.2

NASAL NO AND ALLERGY – A REVIEW

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Abstract

Measurement of nasal nitric oxide (nNO) is attractive since it is completely non-invasive and can easily be performed. The measurements may be useful in the early diagnosis of patients with chronic airway disorders such as Kartager's syndrome and cystic fibrosis. The possible use of nNO measurements in the diagnosis and treatment of allergic rhinitis however, needs to be further evaluated as the findings of nNO concentrations in this disease are variable and contradicting. In this review we will discuss origin, production and measurement of nNO as well as the effect of allergic rhinitis, nasal allergen challenge and medication on nNO concentrations. Subsequently, we examine the published data on nNO values in allergic rhinitis and summarize the effects of treatment of rhinitis on nNO. Eventually, we discuss the potential future role for nNO in the diagnosis and management of allergic rhinitis.

Introduction

Nitric oxide (NO) was initially described as a an endothelium derived relaxing factor due to its action as a vasodilator [1, 2]. We now know that NO has a regulatory role in a wide variety of cellular functions and tissues. Gustafsson et al. [3] first demonstrated that NO is present in exhaled air. Alving et al. [4] observed the presence of NO in the nasal cavity in even much higher concentrations compared to the lower airways. In addition, it was demonstrated that nasal NO (nNO) is affected by inflammation of the upper airways [5-8]. These findings lead to the question whether nNO can be used in a clinical setting as a diagnostic tool of airway inflammation, or more specific of allergic rhinitis.

The applications of nNO measurements in practice are still limited. This is mainly the result of the lack of consensus on measurement techniques, consequently leading to different findings of nNO concentrations in different airway illnesses as sinusitis, nasal polyps and (allergic) rhinitis. Exceptions are primary ciliary dyskinesia [9] and cystic fibrosis [10, 11]. It is well known that the nNO levels in these diseases are low, independent of measurement method. Here nNO can be used as a non-invasive screening tool. In this review we try to answer the question if nNO is helpful in the diagnosis and management of patients with allergic rhinitis.

Origin of nasal NO

Nitrogen is unique among the elements in forming no fewer than 7 molecular oxides, all thermodynamically unstable. NO may react by electron gain to form nytroxyl anion NO^- and by electron loss to form NO+, the nitrosonium molecule [12]. In biological systems the mode and rate of NO metabolism is dependent on its own concentration, its diffusibility and the surrounding concentration of other bioreactants [13-15]. The mode and rate of NO degeneration also varies in the gaseous and the aqueous phase; NO produced by airway epithelium is quickly metabolized in oxygenated aqueous environments by the binding of intravascular haemoglobin, leading to rapid removal of NO from the lungs without systemic side effects [12]. In vivo NO is formed by several isoforms of the enzyme NO synthase (NOS), using the amino acid L-arginine and oxygen (O_2) as a substrate [16, 17]. Different isoforms of NOS exist [16]. The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutively expressed and calcium dependent. Inducible NOS (iNOS) is calcium independent and is expressed in an inflammatory context, in response to certain cytokines or bacterial lipopolysaccharides. Cells potentially capable of generating nitric oxides in the lungs include macrophages [18, 19], neutrophils [20], vascular smooth cells, endothelial cells and pulmonary epithelial cells [2]. On the other hand, the exact origin of nitric oxides in the nose is not exactly known. Most studies indicate

that the main production of nNO is within the mucosal epithelium of the paranasal sinuses [21-23].

Nasal NO: production in health and in allergic rhinitis

There are several studies assessing the difference in expression of NOS in human nasal epithelial cells between normal subjects and patients with allergic rhinitis. Kulkarni et al. [24] localized immunoreactivity for nNOS in healthy humans in the olfactory mucosa, in olfactory receptor neurons, in numerous nerve fibers, in glands, and in surface epithelial cells. Olthoff et al. [25] localized nNOS immunoreactivity in healthy persons and in patients with allergic rhinitis, in the muscular layer of nasal vessels, in the basal portion of submucosal glands, in the periost and in the osteocytes of the inferior turbinate bones. They also found elevated nNOS immunoreactivity around glands in patients with allergic rhinitis. Takeno et al. [26] found that nasal epithelial cells of allergic patients overall produce higher levels of NO through concomitant expression of different isoforms (i and eNOS). Kawamoto et al. [27, 28] also found an increased expression of iNOS in epithelial cells of allergic patients. In accordance, others [29] have shown a higher iNOS expression in allergic patients as well, especially in the submucosal glands of the nose. The increased iNOS expression of epithelial cells in patients with allergic rhinitis may likely be explained by an increase in iNOS activity due to persistent mucosal inflammation [28].
Moreover, Andersson et al. [30] demonstrated that an ostial occlusion as seen in upper airway allergy or infection, caused by mucosal swelling, resulted in a slowly increasing negative pressure inside the sinus cavity. In parallel, the oxygen pressure in the sinus will decrease, resulting in hypoxia, which in its turn is a powerful inducer of NOS. The group concluded that reduction in sinus pressure therefore might result in an increase of nNO production.
Another source of nNO in allergic rhinitis was showed by Hanazawa et al. [31]. In an experiment they showed a significant increase of nNO in patients with allergic rhinitis after recruitment of eosinophils induced by eotaxin, which causes chemotaxis of eosinophils with a clinically symptomatic inflammatory response of the nasal mucosa. This effect is accompanied by an increase in nNO contributing to oxidative stress.

Nasal NO measurement

The chemiluminescence technique for detecting NO in exhaled nasal air is currently the preferred method of measurement [6, 32]. Measurement of nNO requires generation of airflow through nasal cavities aspirating or insufflating air. This can be done via one nostril while the velum is closed or in parallel e.g. exhaling via one or both nostrils, aspirating via the mouth with air entrained into both nostrils during breath holding, or aspirating from one (figure 1), or both nostrils during breathholding.

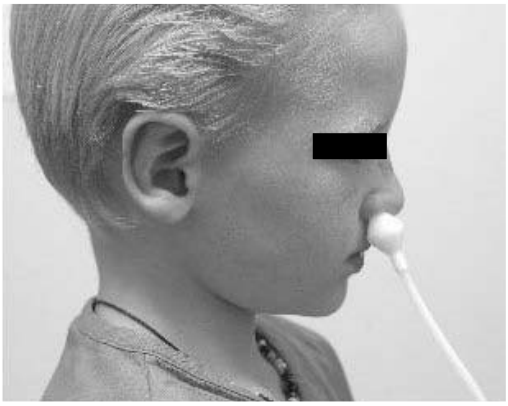

Figure 1.

Example of nasal NO measurement by aspirating air from one nostril during breath-hold.

Closing of the pharynx by the velum is always required to block off oral air. This can be done in a variety of ways. One is, to exhale against a resistance, another is breathholding with the velum elevated, or by voluntarily elevating the soft palate [6]. With all these methods constant transnasal flow produces a washout phase followed by the establishment of a steady NO plateau (figure 2). The nNO concentration is inversely related to the transnasal airflow [6, 33] and thus the aerodynamics of the flow effect the nNO. Besides nNO measurement during quiet exhalation, aspiration or during breathhold, nNO can also be measured while humming [34-37]. Humming is an exhalation while phonating the "m". In this case there is a vibrating or oscillating exhalation flow through the nose. This leads initially to high NO peak levels gradually decreasing to a plateau (figure 3).

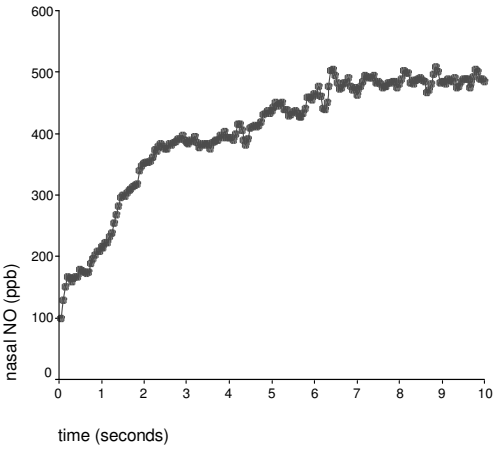

Figure 2.

Example of an on-line nasal NO measurement in a healthy subject during 10 s of breath hold

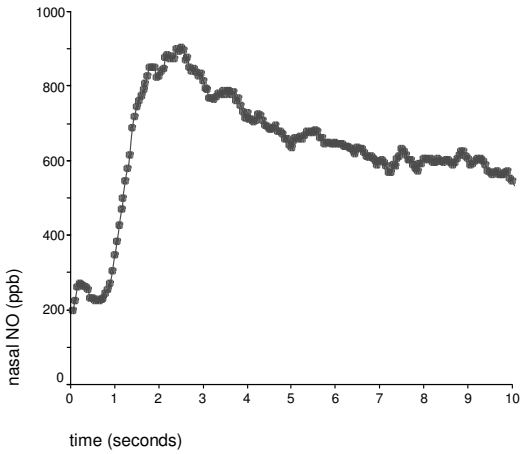

Figure 3.

Example of an on-line nasal NO measurement in a healthy subject during 10 s of humming as loud as possible with the mouth closed.

Nasal NO in healthy subjects and in patients with allergic rhinitis (figure 4)

Kharitonov et al. [38] compared nNO in 46 healthy subjects and in 72 subjects with seasonal allergic rhinitis, some of them had concomitant asthma. The nNO was measured during the pollen season. Seasonal rhinitis was defined by a clinical history of seasonal allergic rhinitis for at least two years. However, skin prick tests were performed and not all allergic subjects were sensitized. NO was measured in one nostril during breathhold with a 0.25 L/min flow. Untreated rhinitis patients with and without asthma had significantly 1.5 fold higher nNO concentrations compared to healthy controls (n=19). With the same method of measurement, Djupesland et al. [33] found, in a study on the aerodynamic influences on nNO output measurements, substantially higher nNO outputs in symptomatic seasonal allergic patients (n=5) compared to healthy controls (n=8) at different aspiration flows (0.2-3.7 L/min). As opposed to Kharitonov and Djupesland, Henriksen et al. [39] found no significant difference in nNO levels (aspiration method) in the pollen-season between controls (n=12) and allergic rhinitis subjects (n=46) (figure 4). There was also no difference between only seasonal (n= 19) and both seasonal and perennial sensitization (n=27). The nNO concentration was also measured in a smaller group during the pollen season, all subjects were symptomatic and on treatment with antihistamines. They found an increase in nNO but this was not statistically significant.

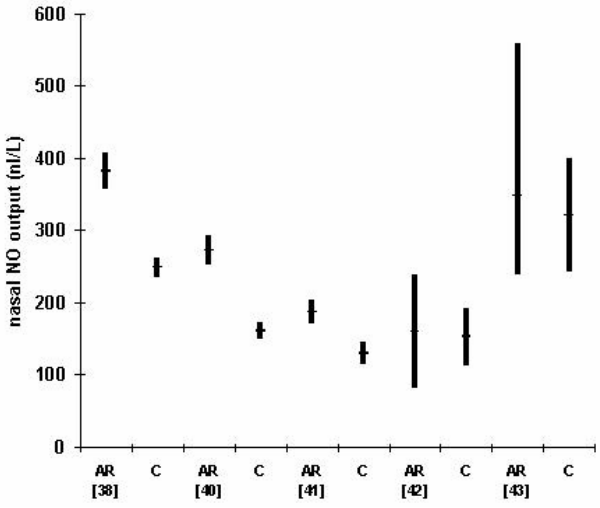

Figure 4.
Mean nasal NO output (with range) in allergic rhinitis (AR) versus healthy controls (C) reported by different authors [38, 40-43]

When comparing baseline nNO concentrations between allergic patients and controls in intervention studies we found that Arnal et al. [40] found significantly higher nNO concentrations in patients with perennial and/or seasonal rhinitis (n=36) with and without active symptoms compared to healthy controls (n=19). The nNO was measured from each nostril during breathhold with an aspiration flow of 0.7 L/min. Another group [41] measured nNO in 21 children with perennial rhinitis out of therapy for at least three weeks. The nNO was measured during aspiration flow of 0.7

L/min from one nostril during 25-s breathhold, which seems quite long for children. Basal mean nNO concentrations in the nose were significantly higher in the affected children compared to the healthy controls (n= 21), 267 ppb (+/- 18) versus 186 (+/- 15) respectively. There was no significant correlation between nasal symptoms and nNO values. In contrast [39, 42, 43] two studies showed that there are no differences in nNO concentrations at baseline in allergic patients compared to healthy controls. Both studies used the same measurement method (aspiration during breathhold), but employed different aspiration flows.

Of interest is the report by Moody et al. [44]. They analyzed the association of nNO and skin prick reactivity to house dust mite. Sixty-four non-asthmatic Pacific Islanders were skin prick tested for 8 common allergens: 56.3% were positive for house dust mite, 25% for cockroach and 12.5 % for grass pollen. The sensitivity of house dust mite reactivity was independently associated with higher nNO concentrations. There was no relation between nNO and a positive skin prick tests for other allergens.

Humming during nNO measurement initially increases nNO levels considerably in healthy subjects [34, 35]. This increase is abolished in patients with an obstructed ostiomeatal complex including those with allergic rhinitis, nasal polyps and sinusitis. The postulated hypothesis is that humming causes a rapid washout of NO from the sinuses. Indeed, such a mechanism was confirmed by a model study [36]. One study indicated that absence of a nNO peak during humming is associated with endoscopic findings suggestive of sinus ostial obstruction in patients with allergic rhinitis [45].

Nasal NO and nasal allergen challenge

There are two studies on the effect of allergy challenge with grass pollen on nNO. Kharitonov et al. [38] studied five seasonal allergic rhinitis patients outside the pollen season. One hour after the start of the challenge when symptoms were at a peak there was a maximal decrease in nNO concentration. Nasal NO concentrations were back at baseline after four hours. Another study challenged nine seasonal allergic patients outside the pollen season [46]. Nasal challenge was done with increasing concentrations of grass pollen extract in each nostril. The challenge did not modify the nNO levels. There is no information on nasal symptoms. Both studies measured nNO from one nostril but with different flows (0.25 L/min and 0.7 L/min). The difference in the effect of the challenge on the nNO concentration might depend on the administered concentration of allergen extract. In Kharitonov's study, it can be assumed that administering a high dose of grass pollen extract all at once will have a larger potential leading to blockage of sinuses due to mucosal swelling and thus influencing nNO compared to administrating a lower total allergen dose in several

steps as in Maniscalco's study. The differences in allergen administration might also influence these findings.

Nasal NO and medication in allergic rhinitis

Palm et al. [42, 43] explored the effect of topical administration of L-NAME, an NOS inhibitor, in subjects with allergic rhinitis. As the expression of NOS in allergic patients is increased [25-29] it seems fair to expect that L-NAME will reduce nNO. The results showed a decrease of nNO in the nose of both allergic patients (n=18) and healthy controls (n=18). The decrease following the L-NAME was larger in patients with the highest base-line nNO levels and higher in all patients compared to controls. However, the dose of L-NAME (50 mg) was rather high and only resulted in about 26-37% decrease in nNO. These findings are similar to what has been observed in two other studies using similar doses [22, 43].
Arnal and co workers [40] compared nNO in allergic subjects before and after administration of a nasal vasoconstrictor. Some subjects with pollen allergy were measured during the pollen season, whereas other patients were tested outside the pollen season. Allergic patients without symptoms had higher mean nNO levels than patients with symptoms. Accordingly, there was an inverse correlation between mean nNO and symptom score. After inhalation of a vasoconstrictor, nNO increased in allergic patients with symptoms, but decreased in the group without symptoms leading to similar high NO levels after vasoconstriction in both patient groups. This can be explained on the basis of sinus obstruction, as the decongestant in the symptomatic patients resulted in an increase in nNO. There was no correlation between nNO and symptom score after nasal vasoconstriction. In healthy controls, nNO decreased with about 25% after a nasal vasoconstrictor. The hypothesis that sinus obstruction results in lower nNO values is supported by the study from Colantonio et al. [47]. They explored nNO values in allergic patients with and without nasal polyps. The nNO values were significantly lower in patients with polyps and the NO levels correlated positively with the extent of the polyposis. Arnal et al. [5] also found lower nNO levels in subjects with nasal polyposis but this was only in case of nonallergic polyposis. Healthy controls and subjects with allergic polyposis had similar nNO values. The nNO concentration in untreated nonallergic polyposis (no steroids) was significantly higher in untreated allergic polyposis. Humming nNO values in a study by Maniscalco et al. [35] showed that post humming nNO output was lower in healthy subjects and subjects with allergic rhinitis than in subjects with allergic nasal polyposis. The described findings suggest that nasal allergy results in higher nNO levels independent of nasal obstruction or ostium obstruction. However, this is not confirmed by nasal allergen challenge (see above).
Antihistamines in allergic rhinitis patients [41, 48] do not affect nNO in subjects with perennial or seasonal allergic rhinitis. Similarly leukotriene antagonists did not influence nNO [49] in seasonal allergic rhinitis. On the contrary there are some studies that showed that topical administrations of nasal steroids do affect nNO [38,

41, 49, 50] by decreasing the nNO concentration. This might be the result of down regulation of the transcription of iNOS [51].

Conclusion

The nNO concentration seems to be elevated in patients with allergic rhinitis in some studies but is similar in others, in comparison with healthy controls and seems to be modified by corticosteroids. Hence, there is no unambiguous answer to the question whether nNO reflects allergic rhinitis and whether there is a future role for nNO in the diagnosis of this disease. It is surprising to us that there are no clear reports on possible apparent difficulties when measuring nNO during (allergic) rhinitis. After all, if there is a certain amount of retained mucous and/or mucosal swelling in the nose and thus the accessibility is limited. Measurement of nNO with the aspiration technique will result in a vacuum and thus impaired measurement.
In summary we are of opinion that collective standardization of nNO measurement and further studies in well-defined populations are imperative to know whether there is a future role for nNO in the diagnosis and management of patients with allergic rhinitis.

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PART 2

NASAL NO MEASUREMENT

CHAPTER 2.1

NASAL NO MEASUREMENT BY DIRECT SAMPLING FROM THE NOSE DURING BREATHHOLD: ASPIRATION FLOW, NASAL RESISTANCE AND REPRODUCIBILITY

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Struben VMD, Wieringa MH, Mantingh CJ, de Jongste JC, Feenstra L. Nasal NO measurement by direct sampling from the nose during breathhold: aspiration flow, nasal resistance and reproducibility. Eur Arch Oto-rhino-lary. 2006 Aug; 263(8):723-8.

Abstract

Objective: assessment of the effect of aspiration flow, the nasal cycle, and time of the day on nasal nitric oxide (nNO) concentrations in air sampled from one nostril during breathhold. *Methods*: nNO was measured in 45 healthy subjects (19 males, aged 18-45 years) from one nostril during breathholding. We compared nNO values and time to plateau in both nostrils with three aspiration flows (0.28; 0.7 and 1.2 L/min) and assessed the short term and long term reproducibility. *Results*: mean nNO values at flows of 0.28; 0.7 and 1.2 L/min differed significantly ($p < 0.01$): 854, 474, 380 ppb respectively. The (median) plateau was reached after 6, 4 and 3 seconds for the different flows. The within-subject coefficient of variability was always < 5%. We found no difference in nNO between left-, right-, largest or smallest nostril ($p > 0.10$). nNO values after 6, 24 hrs and 7 days were not significantly different from baseline ($p > 0.10$) and showed fair reproducibility. The highest aspiration flow was experienced as unpleasant.
Conclusion: nNO can be measured in either nostril and shows no diurnal variation. The measurement is quick, reproducible, feasible and best accepted with an aspiration flow of 0.7 L/min during breathhold for 10 seconds.

Introduction

Nitric oxide (NO) is a gas produced in the airways where it plays a role in the regulation of a wide variety of airway functions [3, 8, 19, 20, 26-29, 35]. High concentrations of NO have been detected in the nose and paranasal sinuses. It has been suggested that the nasal mucosa is the main site of this NO production in the airways [3, 27]. The production of nasal NO (nNO) is influenced by airway diseases as allergic rhinitis, sinusitis, nasal polyps and also by cystic fibrosis and primary ciliary dyskinesia [4, 8, 10, 11, 25, 26, 31, 39].
There are two main approaches to measure nNO. Firstly, infusion of NO-free air into one nostril at a certain aspiration flow while extracting air from the other nostril, where it is analysed, during mouth breathing or breathholding [22]. Secondly, direct sampling from the nose with an air stream generated by the analyser, during mouth or nasal breathing or breathholding [22]. With both methods, a constant transnasal aspiration flow produces an increase followed by establishment of a steady state NO plateau. The nNO concentration is inversely related to transnasal aspiration flow [13]. A commonly used technique is collecting air (passively extracted) with a constant flow from one nostril during breathhold [5, 10-12, 16, 17, 21, 23, 24, 30, 32, 33, 38, 40-42]. The aspiration flows used with this measurement technique vary which results in a wide variation of reported nNO values [13, 14, 18, 33, 36]. Different aspiration flows also resulting in different times of plateau onset which consequently leads to differences in the necessary length of breathhold. The latter seems significant (from a clinical and practical point of view), especially when applying this type of nNO measurement in children [37]. Change of nasal resistance i.e. nasal cycle and the time of measurement might as well influence nNO values. Additionally, there are also many exogenous factors which affect nNO concentrations, such as upper airway inflammation, age, smoking, drugs and otolaryngological surgery [4, 15, 20, 23, 25].
In this study we aim to assess the effects of three different aspiration flows on nNO measurement with direct sampling from the nose during breathhold. In concordance we assessed the effect of the different aspiration flows on the time of onset of the steady state NO plateau. We also examined the effect of nasal resistance on nNO, the reproducibility of nNO after 6 and 24 hours and after 1 week, as well as the within subject variability.

Material and Methods

Study subjects
We recruited 45 healthy non-smoking volunteers. Exclusion criteria were recent (< 3 months) operation in the head-neck region, recent respiratory infection (< 1 week) and known allergy, asthma or other (chronic) airway disease. Use of medication such as inhalation corticosteroids and nasal decongestives that might affect nNO were also exclusion criteria.

Nasal NO measurement
The measurements were performed according to European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines [1, 2, 22]. Nasal NO was measured with a chemiluminescence analyzer (NIOX, Aerocrine, Solma, Sweden). The intrinsic sampling flow was approximately 0.28 L/min. Calibration of the equipment was performed at least every 14 days using 100% nitrogen to zero and then with a certified calibration gas (2120 parts per billion (ppb)). The NO signal output was sent to a computer data acquisition program (NIOX, nasal mode), that displayed real-time measurements in parts per billion (ppb).

Sampling nasal NO
Nasal NO was measured during breathhold after a deep inspiration. The subjects were asked to take a deep breath and hold it for 10 seconds. An NO-inert olive was fitted against either the left or the right nostril. The olive was connected to an adjustable vacuum pump (custom made), which could be set at different aspiration flows. From a side port a sampling tube was led to the NIOX. Measurements were done 'online' which made it possible to detect whether there was a sudden drop in NO concentration resulting from contamination from the lower airways. Thus swallowing or breathing was immediately shown on the on-line curve. In preliminary experiments we measured CO_2 in nasal air as a marker of lower airway contamination. The results confirmed that the NO signal was indicative for velum closure. The average nNO concentration between 7 and 10 seconds after breathholding was recorded. The maneuver was performed in triplicate for each measurement condition. Before every measurement the ambient NO concentration was recorded.

Effects of different aspiration flows
The default aspiration flow of the NIOX is about 0.28 L/min. To generate higher flows we developed a custom made pump device. We measured nNO with the intrinsic flow of 0.28 L/min, and with aspiration flow of 0.7 L/min, and 1.2 L/min. The within-subject coefficient of variability for the 3 flows was assessed. Furthermore the time of onset of the plateau was noted, as the time point where the nNO signal became stable within 20 ppb.

Comparison of right versus left nostril
Prior to the measurements subjects were asked to exhale through the nose on a small mirror to establish the widest (low resistance) versus the narrowest nostril (high resistance). Thereafter we assessed the nNO concentrations in the left and the right nostril. These measurements where assessed with an aspiration flow of 0.7 L/min.

Reproducibility
We assessed the within-subject reproducibility of nNO at t=6 hours, t=24 hours and t=1 week, with a nasal aspiration flow of 0.7 L/min.

Statistical methods
We calculated the average of triplicate measurements and standard deviations. In case of normal distribution of the nNO concentrations, we used paired t-tests for comparing results with the different aspiration flows and left-right comparison. Intra-subject variability was assessed by calculating the within-subject coefficient of variability and reproducibility was assessed according to Bland and Altman [7]. All analyses were done with SPPS, version 10.1 for Windows.

Results

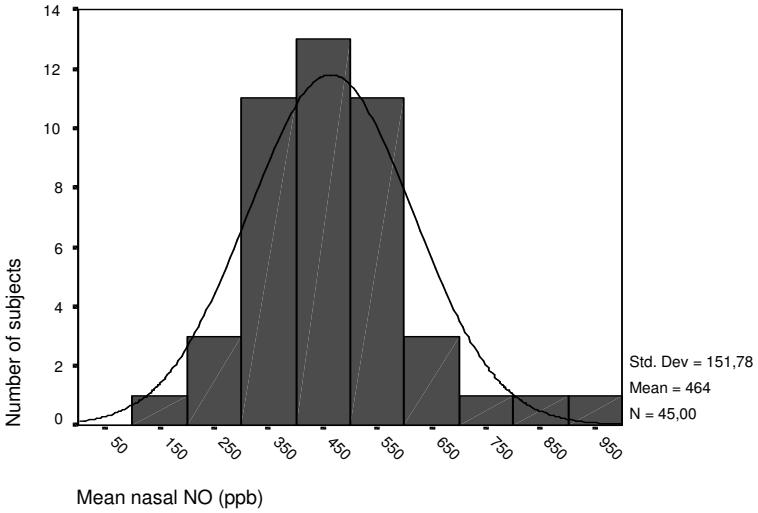
A total of 45 subjects (19 male), median age 26 (18-45), participated (table 1). All subjects were able to cooperate with the tests and had no difficulty holding their breath for 10 seconds. Nasal NO values were normally distributed for a both flows of 0.7 L/min (figure 1) and 1.2 L/min. Invalid measurements, such as an unstable plateau, breathing or swallowing during the measurement, were detected online. These data were discarded and the test was repeated. Ambient NO ranged between 0.2 and 62 ppb, 85% of the measurements were done at an ambient NO < 20 ppb. There was no significant relation between nNO and ambient NO.

Table 1. Characteristics of healthy subjects (n=45)

Male / female	16 / 29
Age (range)	26 (18-45)
Passive smoking	6
Length in cm (range)	1.76 (1.54-1.91)
Weight in kg (range)	68 (40-95)
BMI (range)	22 (16-28)

Effect of different aspiration flows
The mean nNO output at different aspiration flows was assessed in 35 subjects. At an aspiration flow of 0.28 L/min the mean (SD) nNO concentration was 854 (223) ppb, at 0.7 L/min it was 474 (121) ppb and at 1.2 L/min it was 380 (100) ppb (all significantly different $p < 0.001$). Most volunteers tolerated the measurements with 0.28 and 0.7 ml/min flow well. However, at 1.2 L/min many subjects reported unpleasant sensations in the nose.
The median (range) time of onset of the plateau phase was 6 (3-9), 4 (1-7) and 3 (1-7) seconds for 0.28 L/min, 0.7 L/min and 1.2 L/min respectively. For the 0.7 and 1.2 L/min flow there was always a plateau reached within 7 seconds. The within-subject coefficient of variation for the 3 different flows was smaller than 5%.

Figure 1. Normal distribution nasal NO concentration (in ppb).



Nasal NO in left versus right nostril
The mean nNO concentration in the left versus right nostril was assessed in 45 subjects. The mean (SD) nNO concentration sampled from the left nostril was 465 (137) ppb versus 466 (156) ppb from the right nostril (p=0.85). The mean nasal concentration in the widest nostril was 464 (152) ppb versus 467 (141) ppb in the narrowest nostril (p=0.69).

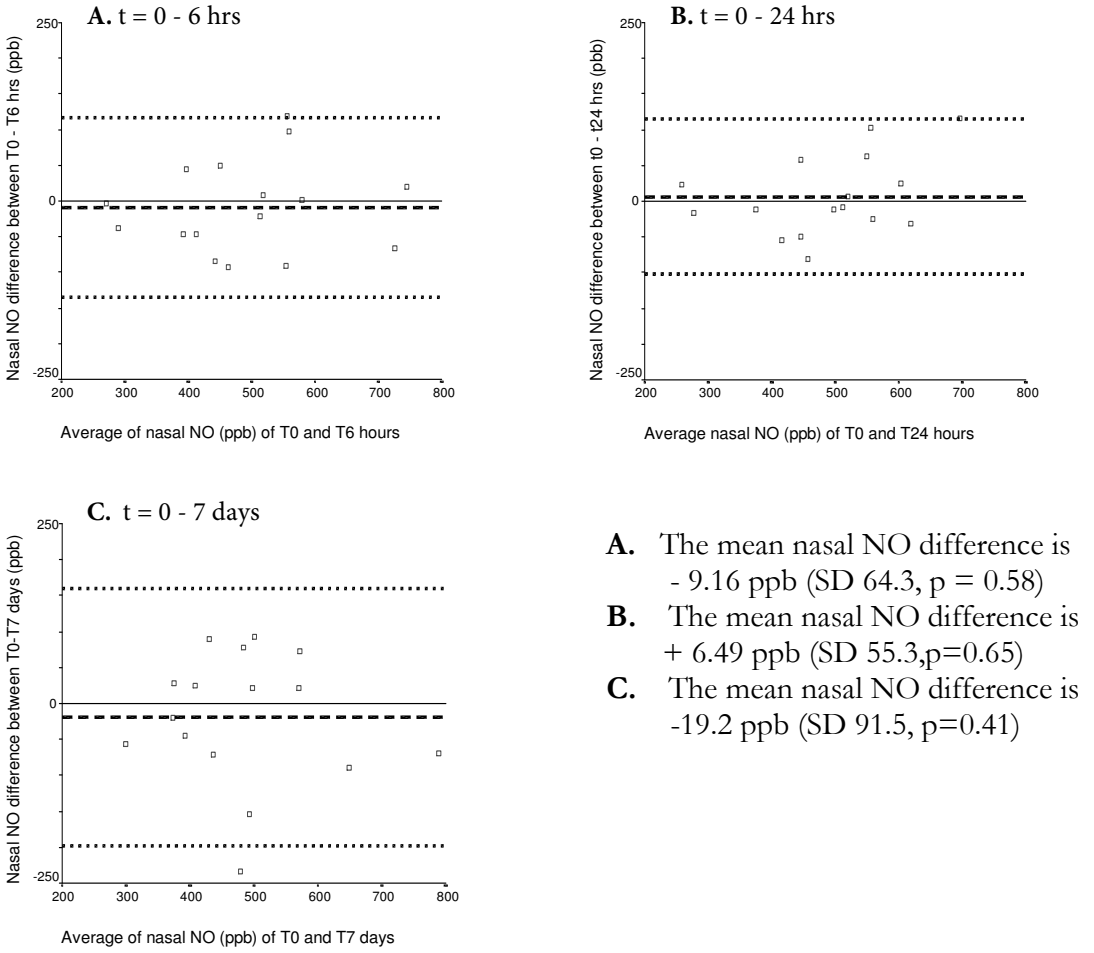
Reproducibility
Within-day and between-day variation in nNO levels (at a sampling rate of 0.7 L/min) was quantified in 16 subjects. There was no difference (p=0.58) between initial mean NO (SD) (487 (139) ppb) and the NO value at 6 hours (496 (132) ppb). Subsequently there was no systemic difference in NO concentrations (SD) between the initial measurement, the measurement at 24 hours (483 (110) ppb) and the measurement 1 week later (493 (132) ppb, p=0.65, p=0.41 respectively). (figure 2 A - C). The difference was independent of the average nNO concentration.

Discussion

In this study, we found that nasal NO (nNO) measurements using passive aspiration flows of 0.28, 0.7 and 1.2 L/min were feasible. However, with a sample flow of 0.28 /min the steady state NO plateau phase was not always reached within 10 seconds and the mean nNO variation was large. Values obtained with an aspiration flow of 0.7 and 1.2 L/min flow were normally distributed and had the advantage of reaching a plateau within 7 sec of breathhold. Yet, a flow of 1.2 L/min caused an unpleasant sensation in the nose. Therefore we selected 0.7 L/min as the preferred flow. We found no

difference of nNO between the left versus right nostril or larger versus smallest nostril. The short- and longtime reproducibility of the nNO concentrations was good.

Figure 2. Bland-Altman plots of mean nasal NO in ppb (horizontal axis) vs difference at specified time points (vertical axis)



A. The mean nasal NO difference is - 9.16 ppb (SD 64.3, p = 0.58)
B. The mean nasal NO difference is + 6.49 ppb (SD 55.3,p=0.65)
C. The mean nasal NO difference is -19.2 ppb (SD 91.5, p=0.41)

Qian et al. [33] assessed the optimal aspiration flow for nNO measurements in 16 adults, from one nostril while expiring orally against a resistance. Steady state NO plateau was defined as a variation of NO concentration less than 5 ppb over a period of at least 10 seconds. They used the aspiration flows of 0.9; 2.2; 3.2; 4.2; 5.2; and 6.2 L/min and calculated the nNO output (= flow (L/min) x NO (ppb)) corrected for body surface area (M^2). None of the subjects achieved a stable plateau of nNO concentration at a flow of 0.9 L/min. Several subjects had difficulties reaching a plateau with flows of 5.2 and 6.2 L/min due to flow induced alar collapse. There was no significant difference in nNO using flows ranging from 2.2 to 6.2 L/min, the mean output corrected for body surface was 300.7 nl/min/M^2 (= 550 nl/min). The authors conclude that the optimal range of aspiration flow is 3.2 –5.2 L/min in adults. This is

in agreement with the recommended aspiration by the ATS [1, 2] and is due to the fact that nNO outputs found at a transnasal airflow of <1 L/min are substantially smaller than those at > 2 L/min [13] resulting in an underestimation of NO outputs obtained from low flows (< 1 L/min) compared to output at higher aspiration flows. However, lower aspiration flows are used in a majority of studies on nNO (< 1L/min) [10-12, 16, 17, 21, 23, 24, 30, 32, 33, 38, 40-42] to asses the NO values.
The previous persuaded us to assess nNO at lower (clinically more practical) aspiration flows. In agreement with Qian and others [13, 14, 33] we found lower nNO concentrations at higher aspiration flows as well. We also found, in accordance with literature [13, 14, 18, 33, 36], that at higher flows the steady-state plateau was reached earlier. Compared to Qian et al, we used a less strict definition of a stable steady state NO plateau, therefore subjects reached a plateau more often at lower aspiration rates. Although with our lowest flow (0.28 L/min) frequently more than 10 seconds were required to reach a plateau. Another consequence of a lower aspiration flow is the relatively large standard deviations of the nNO values which results in difficulties when looking for subtle changes in nNO as for example in allergic rhinitis or sinusitis. This in contrast to Bartley et al [6], who found low standard deviations with aspiration flows of 250 and 500 ml/min. Probably this difference is the result of longer sampling times (although not described in the article).
With our highest flow (1.2 L/min) the majority of the subjects experienced discomfort in the nose. We did not quantify this finding, but in children (preliminary results) the sensation resulted in laughing (during) and discontinuation of the measurement.

Another aspect of potential influence on the transnasal airflow is the nasal cycle. Congestion (increased resistance) of one nose cavity reciprocates with decongestion (decreased resistance) of the other. Qian and coworkers demonstrated a significantly negative correlation between nasal cavity volumes i.e. nasal resistance and nNO concentrations [34]. Chatkin et al. on the other hand demonstrated an absence of correlation between the magnitudes of changes in nNO and changes in nasal cavity volume [9]. In our healthy subjects, we could not find an association between the nNO concentration and the widest versus the narrowest nostril as well as between the left versus the right nostril. We conclude that the nasal cycle does not influence nNO concentrations during the measurements of nNO in one nostril.

We found good short- and long term reproducibility of nNO. Silkoff et al. examined the reproducibility of different nNO measurement techniques [36]; nNO measurement with air aspirated from one nostril (3.3 ml/sec) showed excellent reproducibility as well. Bartley et al. found good reproducibility with sampling rates of 250 ml/minute and 500 ml/minute [6].

Our findings suggest that nNO measurements during breathholding can be measured from either the left or right nostril at any time of the day. The measurement is safe, quick and reproducible. The clinical utility of nNO measurements should be assessed

using standard methodology. We propose from a clinical point of view an aspiration flow of 0.7 ml/min, as this was better accepted than a flow of 1.2 L/min and produced a more rapid plateau response than a flow of 0.28 L/min.

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CHAPTER 2.2

NASAL NO: NORMAL VALUES IN CHILDREN AGE 6 THROUGH 17

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Struben VMD, Wieringa MH, Mantingh CJ, Bommeljé C, Don M, Feenstra L, de Jongste JC. Nasal NO: normal values in children age 6 through 17 years. *Eur Respir J, 2005, Sep;26(3):453-7.*

Abstract

Objective: Assessment of normal values of nasal nitric oxide (nNO) in healthy children. *Methods*: Healthy children aged 6-17 from three schools in Rotterdam were recruited. Breath was held for 10 seconds, while air was extracted from one nostril with 0.7 L/min. The mean nNO value at the response plateau after 7-10 seconds was recorded and the average of three measurements was used. *Results*: 340 children participated; the boy/girl ratio was 156/184. Three reliable measurements were available in 85% of the children. Nasal NO concentrations were distributed normally, mean: 449 ppb (SD 115). Nasal NO was not associated with gender (p=0.30), passive smoking (p=0.25) or body mass index (p=0.61). In children < 12 years nNO correlated positively with age ($\beta^* = 11.5$, $p < 0.01$), history of adenoidectomy ($\beta^* = -57.5$, $p = 0.02$) and ambient NO ($\beta^* = 0.50$, $p < 0.01$). In children $\geq$ 12 years, ambient NO was the only significant modifier. Prediction rules for nNO values in children were formulated. *Conclusion*: we present normal values for nNO in children, which can be used to assess the value of nNO in respiratory illnesses.

* Adjusted betas

Introduction

Nitric oxide (NO), a potent biological mediator, was first demonstrated to be present in orally exhaled air by Gustafsson et al [1]. A few years later Alving et al. [2] observed presence of NO in the human nasal airways and in the paranasal sinuses in much higher concentrations compared to the lower airways. Studies in healthy adults indicate that NO in nasal air is mainly produced in the epithelial cells of the nasal cavity, particularly in the paranasal sinuses [3]. NO is involved in the local host defence of the upper airways, acts as an airborne messenger and as a regulator of mucociliary function in the nasal airway [4-7]. In addition nasal NO (nNO) is affected by inflammation of the upper airways [8-12].
Measurement of nNO is easily performed and can be used to screen for disease or to monitor treatment effects. The use of nNO measurements in clinical practice however, is still limited. On the one hand, because the effects of different physiological and pathologic conditions on nNO still needs further research. On the other hand, there is a lack of consensus on measurement techniques, consequently leading to different findings of nNO concentrations in different airway illnesses as sinusitis [10, 13], polyposis nasi [8, 10], and (allergic) rhinitis [9, 14-16]. Exceptions are cystic fibrosis [17-22] and primary ciliary dyskinesia [23-26]. It is well established that the nNO levels in these patients are extremely low, independent of measurement method.
There is only one study on normal values of nNO in healthy children [27]. The assessment of normal values of nNO may be important for determining the role of nNO as a marker of inflammatory disorders of the upper airways. The reported effects of inflammation on nNO are not consistent [10, 13, 25, 28-32]. The conclusions of the various studies may differ because of methodological factors, including different sampling methods, sampling flow-rate and the influence of ambient NO. In previous studies, the effects of airway diseases and treatment on nNO have been compared to normal nNO levels obtained from relatively small control groups which are not suitable to assess normal values, and do not necessarily represent a sample of the general population [11, 14, 33]. We aimed to collect normal values for nNO in a large population of healthy children aged 6 trough 17 years using a method previously validated [34].

Methods

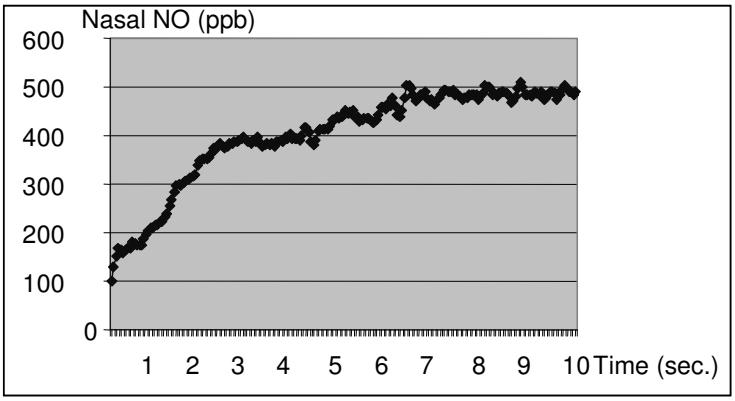
Subjects and setting
Children (age 6 through 17) from 2 primary and 1 secondary school were invited. All parents, and all children older than 12 years, were asked to fill out a questionnaire, based on the ISAAC core questionnaire [35, 36] extended with questions on in- and exclusion criteria and potential confounders (gender, age, height, weight, body mass index, history of ENT surgery and passive smoking). The inclusion criteria were age 6 - 17 years, written consent from the parents and the child itself when older than 12 years. Exclusion criteria were: physical exercise immediately before the NO

measurement, active smoking, allergy and/or asthma (based on ISAAC core questionnaire), airway influencing medication (e.g. inhalation corticosteroids, nasal decongestives), chronic airway disease (e.g. cystic fibrosis, primary ciliary dyskinesia), and recent (< 3 months) adeno- and/or tonsillectomy.

Nasal NO measurements

Nasal NO was measured with a NIOX chemiluminescence analyser (Aerocrine, Solna, Sweden). The air was sampled with a flow of 0.7 L/min from the nostril with the best patency [34]. Calibration of the equipment was performed every 14 days using 100% nitrogen to zero and with a certified calibration gas (NO, 2120 parts per billion (ppb)). The NO signal was sent to a computer data acquisition program (NIOX, nasal mode, Aerocrine, Sweden), that displayed real-time measurements. Nasal NO was measured during breath hold after a deep inspiration. An NO- inert olive was placed firmly against one nostril. The olive was connected to an adjustable vacuum pump (custom made) to obtain a flow of 0.7 L/min [34]. From a side port a sampling tube was led to the NIOX. Subjects were asked to take a deep breath and hold it for 10 seconds. The average nNO concentration was calculated at the plateau between 7 and 10 seconds after breathholding (figure 1). Preliminary experiments indicated that with this technique, the soft palate was closed as evidenced by absence of CO^2 in the aspirated air. Any leakage was evident from an increase in CO^2 and a sudden drop in nNO. The manoeuvre was performed in triplicate. To obtain three correct measurements a maximum of six attempts were made. Before every measurement the ambient NO concentration was recorded. The Ethical Committee of the Erasmus medical centre approved the study protocol.

Figure 1. Example of on-line nasal NO measurement during 10 seconds of breathhold and an aspiration flow of 0.7 L/min.



Statistical methods

Nasal NO concentrations were expressed as the mean of three measurements. For the analysis of the relationship between nNO and potential confounders, univariate analyses were performed. When the univariate analysis appeared to be significant ($p <$

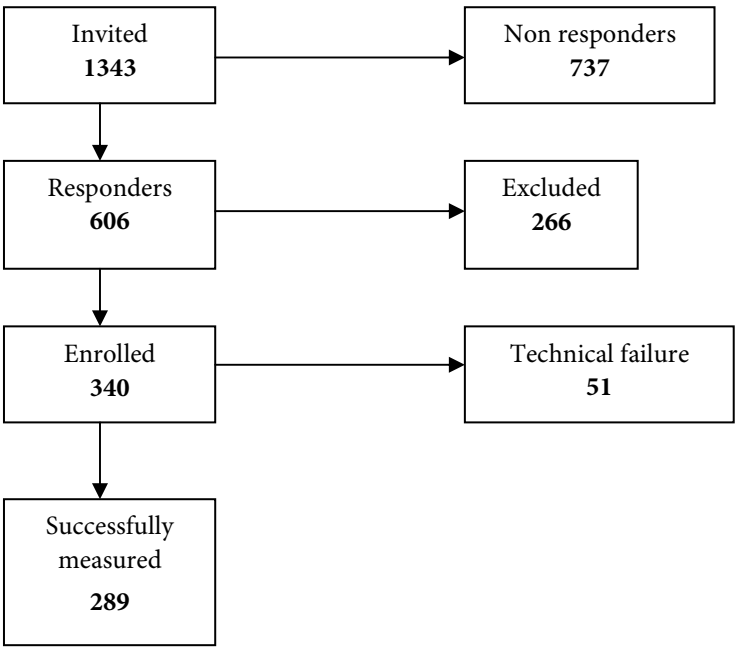
0.10, 90% confidence) we included the variables in a multivariate analysis. For the multivariate analysis we used different linear and quadratic models to test nNO concentration in healthy children as a function of covariates. Algorithms were formulated to predict normal nNO in healthy children by fitting linear and quadratic models. All analysis was done with SPPS, version 10.1 for Windows and in SAS.

Results

Study population

One thousand three hundred and forty three subjects were invited by letter to participate. Six hundred and six children (58%) responded. The response at the secondary school (36%) was much lower than at the two primary schools (61% and 57% respectively). Three hundred and forty children (56%), aged 6 through 17 years met the inclusion criteria (156 male and 184 female) and were enrolled (figure 2). Two hundred and sixty six (44%) were excluded (128 had wheezed, 154 sneezed, 66 smoked, 69 had other health disorders; numbers overlap). Most children were Caucasian (92.4%). The mean (SD) body mass index was 18.5 (3.1). Of all included children 41 (12.1%) had a history of adenoidectomy, but none within 3 months before the measurement.

Figure 2. Flowchart of the study.

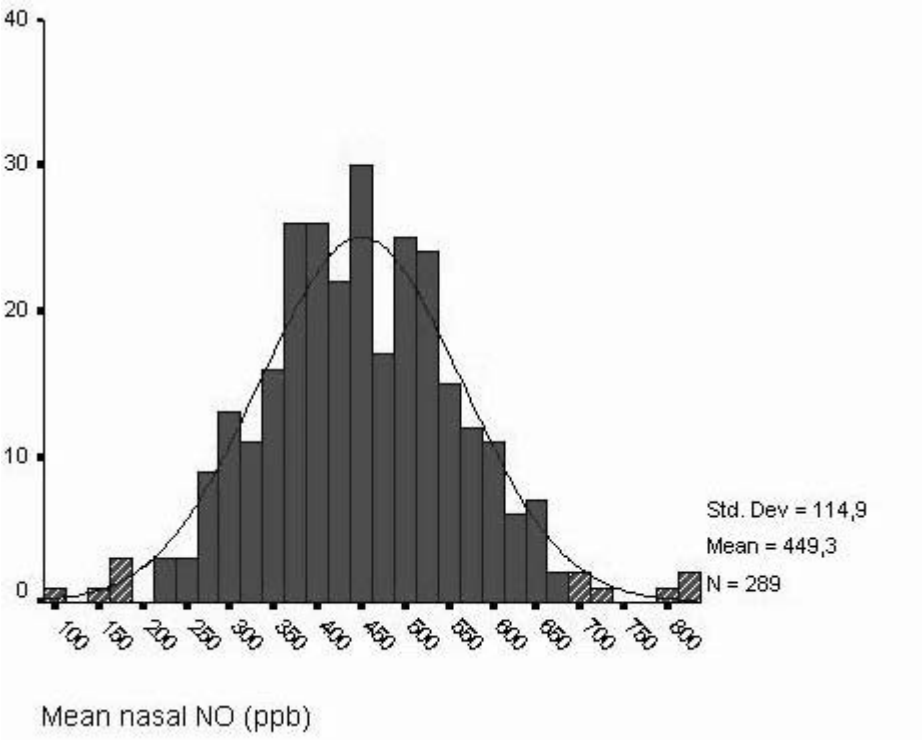


Nasal NO values

Two hundred and eighty-nine children successfully performed the nNO measurements (mean age 11.6 years). Fifty-one (15%) had problems with performing the measurements (mean age 11.2 years) including difficulties in maintaining an

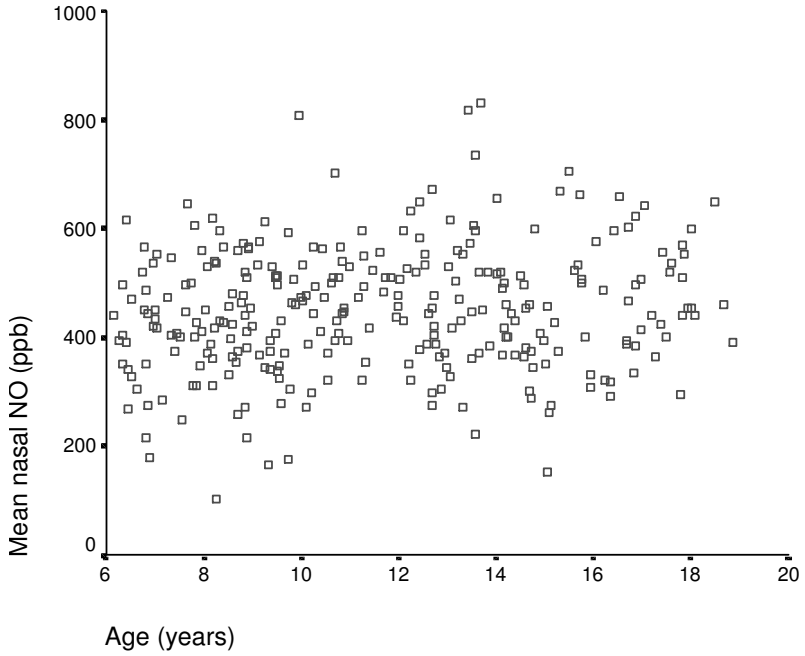
adequate palatal closure and failure to achieve a stable plateau. This group was not significantly different from the whole study group. The values of nNO were normally distributed (mean 449 ppb, SD 115) (figure 3). The ambient NO ranged from 5 - 182 ppb, with a median of 43 ppb. Nasal NO values were independent of gender, passive smoking, height, weight and body mass index.

Figure 3. Distribution of mean nasal NO concentrations (in ppb) in healthy children aged 6 through 17 years. The non-striped area represents 95% of the subjects.



We fitted several models to describe the relationship between nNO and age and other covariates. Plotting nNO against age suggested an increase towards a plateau at older age (< 12 years) (figure 4), therefore a quadratic model was fitted but it appeared not to contribute significantly in describing the relationship. Also a standard linear model did not fit the data sufficiently. Subsequently, a linear model depending on age, with two different slopes connecting at one point was fitted with a non-linear least squares method. The model predicts the intersection of the two slopes at the age of 11.2 years. Taking into account the interaction with age we proposed to stratify for age in two groups in the algorithm for predicting nNO.

Figure 4. Nasal NO values (in ppb) in healthy children by age.



In children < 12 years the mean nNO value correlated positively with age (adjusted β=11.5, p< 0.01) Nasal NO was modified by a history of adenoidectomy in the past (adjusted β= -57,5, p=0.02) and correlated positively with ambient NO (adjusted β= 0.50, p<0.01). These associations were non-significant in children 12 years and older except for ambient NO. We derived two prediction rules for nNO value in healthy children. The equations predict the mean nNO concentration corrected for age in years, history of adenoidectomy (yes = 1 and no = 0) and ambient NO (in ppb) in healthy children (on the condition of the inclusion criteria described in the 'methods').

Prediction rule for nNO age < 12:
nNO = 314.6 + 11.5 * age – 57.5 * history of adenoidectomy + 0.5 * ambient NO

Prediction rule for nNO age $\geq$ 12:
nNO = 452.6 - 2.9 * (age –12) – 16.0 *history of adenoidectomy + 0.5 * ambient NO

For example the approximate nNO concentration of a child aged 10 years that never had an adenoidectomy and an ambient NO of 15 ppb will be: 314.6+ (11.5 * 10) -(57.5 * 0) + (0.5 * 15) = 437.1 ppb.

Discussion

We assessed nNO in a large group of healthy children. In 289 children, aged 6 - 17 years, nNO was normally distributed and depended on ambient NO and, only in those younger than 12 years, on age and history of adenoidectomy. Gender, passive smoking, body mass index, weight and height did not influence nNO. Our study is one of the first studies on normal values of nNO conducted in a large number of healthy children.

In the majority of the clinical studies on nNO, comparisons are made with small 'normal' control groups. There is only one study [27] that formally intended to establish normal nNO values in children. Daya et al. [27] assessed nNO values in 30 healthy children, 18 boys and 12 girls, aged 3.2 to 17.6 years and found a considerable variability, which may be ascribed to the racial heterogeneity (17 Caucasian, 7 Oriental and 6 Negroid children) and the small size of the study group [27]. Besides, there were only 1 to 4 children per age category. The children were recruited from siblings or friends from patients attending an ENT clinic, and may not represent a sample of the general population. Moreover, age and other potential confounders such as ambient NO were not taken into account.
On the basis of literature we excluded children with airway morbidity, recent infections (< 1 week) and recent adeno- and/or tonsillectomy (< 3 months) [3, 8, 10, 11, 13, 14, 16, 29, 30, 33, 37-47]. This also applied for smoking and children using airway-influencing medication. Physical exercise immediately before the measurements was not allowed. Gender, age, height, weight, body mass index, history of adenoidectomy, passive smoking and ambient NO were considered as covariates.
In our study, age was positively associated with nNO. The association was significant below the age of 12. Radiological anatomy of the paranasal sinuses shows that in 12-year-olds sinuses reach their final size [48, 49]. Because of this and in combination with the results of the multivariate modeling we analyzed the data with a break at the age of 12 years. The association between nNO and age in children younger than 12 was approximately 3 times stronger compared to children aged 12 years and older. In the latter group the association was not significant anymore, indicating that age is an interaction factor. These findings are in agreement with the hypothesis that nNO concentrations are correlated with the anatomical development of the paranasal sinuses [48, 49]. An additional factor, explaining the association between age and nNO might also be the increase of the nasopharyngeal airway during pre- and early adolescence while adenoid regresses. The altered volume influences the intranasal flow and, perhaps, nNO concentrations.
Adenoids develop during infancy and reach a maximal size between 2 and 14 years [50]. In our study nNO was significantly lower in children with a history of adenoidectomy. This association was only found in children < 12 years probably because after this age adenoids regress rapidly, making children with an adenoidectomy comparable to children without. Besides, removal of adenoids is more

likely in case of chronic respiratory infections, which might cause elevated nNO [14, 29, 30].

Little is known about how to deal with the influence of ambient NO on nNO. We found a significant relation between ambient and nNO. Ambient NO was about 10% (median 43 ppb) of the mean nNO. So the presented absolute levels and the calculated output (ppb x sample flow) may have been overestimated. This is a problem in the assessment of nNO as a diagnostic tool or monitoring tool in case of for example allergic rhinitis were there might be only subtle changes in nNO, in contrast to, primary ciliary dyskinesia or cystic fibrosis where nNO is much lower than in healthy subjects. Several investigators have simply subtracted ambient NO from nNO [16, 43] without justification. Our data show that ambient NO and nNO are not simply additional but that correction for nNO had to be made by subtracting 0.50 ppb per ppb of ambient NO. The fact that we found a relationship with ambient NO, while others did not, could be explained by the large range of ambient NO values (5-182 ppb) in the present study, whereas in most previous studies ambient NO did not exceed 20 ppb [9, 10, 14, 33].
Further studies in other populations should confirm whether our prediction rules, subtracting 50% of ambient NO, have general validity. For the moment it seems prudent to include healthy controls in any studies exploring nNO in disease.

The present study used questionnaire-based information for inclusion, without physical examination and/or laboratory tests to confirm the health status of the studied subjects. The validity of such findings can be questioned. However, physical examination is not sensitive to detect allergic disease, and the questionnaires used are well validated in the ISAAC study and showed good agreement with objective tests of allergy [35, 36].

In summary, we established a normal reference range for nNO in healthy children, and developed an algorithm to predict normal nNO on the basis of age, ambient NO and a history of adenoidectomy. Establishing the normal range of nNO in 6 to 17 year old healthy children is important for the investigation of the nNO measurement as a screening- or even diagnostic test for various inflammatory conditions of the upper airways like allergic rhinitis, cystic fibrosis or primary ciliary dyskinesia.

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CHAPTER 2.3

SILENT AND HUMMING NASAL NO MEASUREMENTS IN ADULTS AGED 18-70 YEARS

Eur J Clin Invest. 2005 Oct;35(10):653-7.

Adapted from:
Struben VMD, Wieringa MH, Mantingh CJ, Bruinsma SM, de Jongste JC, Feenstra L. Silent- and humming nasal NO measurements in adults aged 18-70 years. *Eur J Clin Invest. 2005 Oct;35(10):653-7.*

Abstract

Background: the concentration of nitric oxide (NO) measured from the nose is much higher than in the lower airways and increases during humming. We assessed nasal NO (nNO) normal values during breathhold and during humming in healthy adults. *Materials and Methods*: nNO concentrations were measured in healthy adults (age 18-70). They held their breath for 10 seconds and thereafter they hummed as loud as possible with their mouth closed also for 10 seconds. During breathhold, air was passively extracted from one nostril with 0.7 L/min. The average NO output at the plateau after 7-10 seconds was recorded and the mean of three consecutive measurements was calculated. During humming, air was extracted with 1.2 L/min, the peak NO values were recorded. *Results*: hundred healthy adults participated (37 males). The nNO concentrations during breathhold were distributed normally (mean: 455 parts per billion (ppb), SD 147). A random subgroup of 40 out of the 100 subjects (15 male) performed nNO measurement during humming. The median peak NO value was 1019 ppb (SD 561) at the first measurement and 837 ppb (SD 408) at the second. There was a significant difference between the peak NO values of 1st and 2nd humming. *Conclusion*: We present normal values for nNO in adults, which can be used to assess the value of nNO in respiratory illnesses. The peak nNO values during humming are variable, and their clinical relevance remains to be shown.

Introduction

Nitric oxide (NO) is a gas produced in many cells of the respiratory tract [1-3]. The amount of NO measured in the nasal airways is much higher compared to the concentrations found in the lower airways [3-6]. Studies in healthy adults indicate that NO in nasal air is mainly produced in the epithelial cells of the nasal cavity, particularly in the paranasal sinuses [3, 7-11]. Nasal NO (nNO) is involved in the local host defence, acts as an airborne messenger and as a regulator in mucociliary function of the nasal mucosa [3, 12]. In addition, nasal NO (nNO) is affected by inflammation of the upper airways.
The assessment of nNO values is easily performed in adults and also in children. Therefore it is hypothesised that nNO might be used as a tool to screen for disease or to monitor treatment effects. However, studies on nNO in healthy subjects and subjects with upper respiratory diseases as allergic rhinitis [9, 10, 13-19], sinusitis [20-25], nasal polyps [20, 26, 27] or mucociliary disorders [28-36] are not yet conclusive regarding nNO as such a potential screening i.e. monitoring tool. There are still a lot of contradicting findings remaining. Several issues can explain these contradictions. For instance, measuring nNO can be performed in different ways [37, 38]. Subsequently, measured nNO values will be influenced by the method, in particular the nasal airflow [39], but also by oscillation of the air that occurs during humming [27, 40-43]. Humming means bringing air into vibration by phonation (that is exhalation) of a nasal pitch as an 'm' or 'n'. The effect of humming on nNO is characteristic, leading to an instant increase in nNO levels at the beginning of the measurement. The effect is only recently discovered [40]. It is postulated that the air, which is oscillating due to humming, results in an increase in the exchange of air between the sinuses and the nasal cavity [27, 40, 42]. The effect of diseases of the upper airways on humming nNO characteristics is not completely clear yet.

More knowledge regarding nNO values will be helpful in further exploring the physiological, pathophysyological and diagnostic role of nNO in the upper airways. Humming might give a new dimension in this exploration. To assess the usefulness of nNO measurement in clinical setting, determination of the normal nNO range is mandatory [44]. In this study we aimed to assess normal nNO values under conditions of breathhold or humming in healthy adults aged 18 through 70 years.

Materials and methods

Subjects
We recruited 100 healthy non-smoking volunteers age 18 through 70 years by means of advertisement in the Erasmus Medical Centre. Exclusion criteria were: active smoking, symptoms of allergy and/or asthma (based on the European Community Respiratory Healthy Survey (ECHRS) core questionnaire [45]), airway influencing

medication (e.g. inhalation corticosteroids, nasal decongestives), chronic airway disease (e.g. cystic fibrosis, primary ciliary dyskinesia), and recent (< 3 months) operations of respiratory tract areas (e.g. sinus surgery, nasal surgery, etc). Confounders including age, height, weight and passive smoking were assessed by questionnaire.

Nasal NO equipment
Nasal NO was measured with a NIOX chemiluminescence analyzer (Aerocrine, Solna, Sweden). For the assessment of normal values during breathhold (silent) air was sampled with a flow of 0.7 L/min from one nostril [46]. Calibration of the equipment was performed every 14 days using 100% nitrogen to zero and with a certified calibration gas (NO, 2120 parts per billion (ppb)). The NO signal was sent to a computer data acquisition program (NIOX, nasal mode, Aerocrine, Solna, Sweden) that displayed real-time measurements.

Sampling nasal NO
Silent nNO values were measured during breathhold after a deep inspiration. An NO - inert olive was placed against one nostril. The olive was connected to an adjustable vacuum pump to adjust the flow to 0.7 L/min. From a side port a sampling tube was led to the NIOX. Subjects were asked to take a deep breath and hold it for 10 seconds. The average nNO concentration at the plateau between 7 and 10 seconds after breathholding was calculated. In preliminary experiments, soft palate closure was confirmed by the absence of an increase in CO_2 during sampling. The manoeuvre was performed in triplicate.

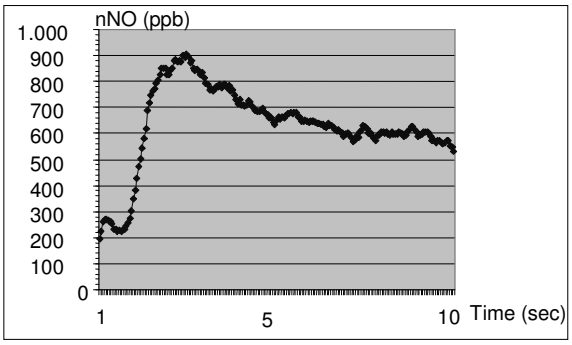


Figure 1. Example of an on-line nasal NO measurement in a healthy subject during 10 s. of humming as loud as possible with the mouth closed.

Subsequently the subjects were asked to take a deep breath and start nasal humming as loud as possible with their mouth closed for ten seconds. The results were recorded continuously in ppb. Preliminary results showed that peak nNO values in about half of the subjects appeared outside the measurement range of the NIOX (0 – 2100 ppb). The NIOX analyzer at that time was to be restricted by its default calibration and could not measure above the calibration range (i.e. 2100 ppb). We solved this technical issue by adjusting the aspiration flow to 1.2 L/min, to keep nNO concentrations within the indicated measurement range [39]. The humming

manoeuvre was performed twice with a 1-minute interval. For every measurement the peak nNO concentration was recorded (figure 1). An nNO peak was defined as at least 1,5 x the average nNO value between 7-10 seconds.
Before each measurement the ambient NO concentration was recorded.
The Ethical Committee of the Erasmus medical centre approved the study protocol.

Statistical methods
For the assessment of nNO normal values we calculated the mean of triplicate measurements, and standard deviations. For the assessment of nNO values during humming we recorded the mean NO in ppb and calculated nNO output (= flow (ml/min) x NO concentration (ppb)) of the peak measurements with the standard deviation and the minimum and maximum values.
If the nNO values during humming showed a normal distribution, we used t-tests for comparing the first and second measurement otherwise non-parametric tests were used. For the analysis of the relationship between nNO concentrations/output and potential confounders univariate analyses were performed. When the univariate analysis appeared to be significant ($p < 0.10$, 90% confidence) we included the variables in the multivariate analysis. For the multivariate analysis we used linear regression models to test nasal NO concentration in healthy adults as a function of potential confounders. All analysis were done with SPPS, version 10.1 for Windows.

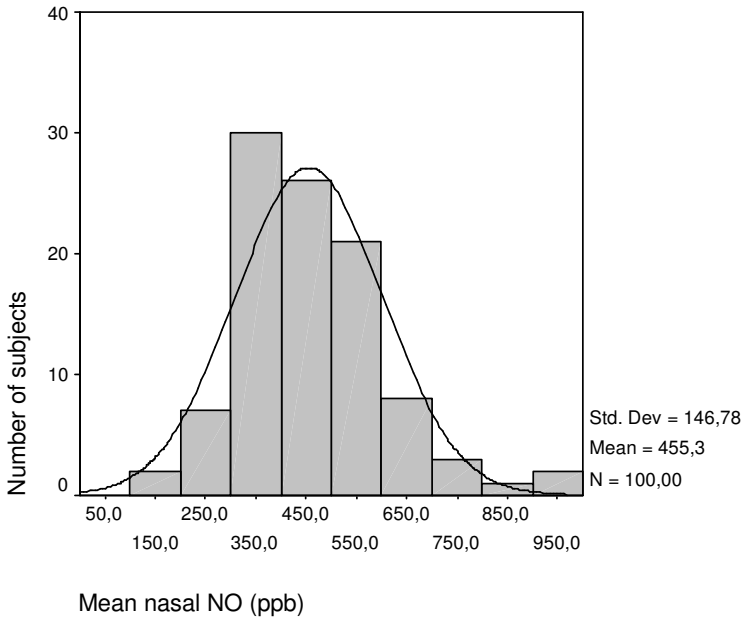
Results

Nasal NO normal values during breathhold
A total of 100 subjects (37 male), median age 30 years, participated (table 1). Silent nNO values were normally distributed (mean 455 ppb, SD 147) (fig 2). The ambient NO ranged between 0.2 and 62.0 ppb, with a median of 3.7 ppb. There was no significant relation between nNO and ambient NO. Nasal NO values were not significantly correlated with age, gender, weight ($p = 0.07$) and passive smoking. The mean nNO value was positively associated with height (adjusted ß = 510, $p = 0.01$).

Table 1. Characteristics of healthy subjects age 18 through 70 years (n=100)

Male / Female	37 / 63
Age (SD, range)	36 (15, 19 – 76)
Passive smoking	13
Height in cm (SD, range)	175 (0.09, 154 –197)
Weight in kg (SD, range)	71 (13, 40 – 110)

Figure 2. Distribution of nasal NO concentrations (in ppb) during breath hold in healthy adults age 18 through 70 years.



Humming nasal NO values

At random 40 out of the 100 subjects (15 male), mean age 47 years, range between 22 and 76 years, performed both silent and humming nNO measurements. All subjects were able to cooperate with the tests and had no difficulty humming for 10 seconds. Nine subjects (23%) had nNO peaks above 2100 ppb, and were excluded from the analyses. We found a characteristic flow curve (figure 1) with a peak followed by a slow decrease towards a plateau. There was a peak visible in 81% of the measurements. The peak nNO values were not normally distributed. The remaining 19% did not have a peak value defined as at least 1,5 times the average nNO value between 7-10 seconds. These subjects did show an increase in NO during humming but without a convincing nNO peak as defined. The median nNO at the peak during the first humming measurement was 1019 ppb (SD 561), minimum 200 ppb, maximum 2100 ppb, median nNO output 1223 nl/min. At the second humming measurement the median NO at the peak was 837 ppb (SD 408), minimum 120 ppb, maximum 1820 ppb, median nNO output 1004 nl/min. The nNO values at the peak were significantly smaller during the second humming manoeuvre ($p < 0.02$). The plateau value (between 7-10 secs) was 386 ppb (SD 186) at the first measurement and 293 ppb (SD 133) at the second measurement. There was no significant difference between the plateau during silent measurements and humming measurements ($p=0.24$). There was no significant association between the humming nNO at the peak and age, passive smoking, gender, height and weight.

Discussion

We established the normal nNO values in adults, assessed during breathhold using an aspiration flow of 0.7 L/min. The mean nNO value for adults was 448 ppb. This value is positively influenced by height. Humming during nNO measurements causes an initial peak in nNO in healthy adults, which diminishes after repeated humming.

Bartley et al. [47] obtained normal nNO values in 37 healthy European non-smoking volunteers, age 21 to 57 years. They found a mean nNO concentration of 436 ppb with a flow of 500 ml/min, and an nNO output (nNO in ppb x flow L/min) of 218 nl/min. A continuous sample was aspirated during an undefined period of breathholding. The NO levels were unrelated to age and gender and all measurements were taken at an ambient NO < 20 ppb. We found a slightly higher NO level at a flow of 0.7 L/min (455 ppb, output = 319 nl/min). The different results might be explained by different type of NO analyzer used. Also the possible difference in time of breathhold might explain the difference.

Recently, it has been shown that humming increases nNO [40]. It is believed that this is due to the oscillating airflow produced by humming, enhancing sinus ventilation and thereby increasing nNO levels. Single-breath humming causes an initial peak in nNO output, followed by a progressive decline. There is no consensus on methods of nNO measurements during humming. Only a limited amount of studies on nNO during humming are available [27, 40-43]. In these studies the nNO output was measured during oral and nasal single-breath exhalations. A tight-fitting mask covering the nose was used for nasal measurements and a mouthpiece for oral exhalations.

With our method we confirmed the characteristic flow curve (figure 2) and lower nNO peak values after repeated humming [27, 41-43]. We used an olive placed against one nostril. Subjects were asked to inhale as deep as possible and thereafter start humming (nasal exhalation) as loud as possible meanwhile extracting air from the nose with a sampling rate of 1.2 L/min. Silent nNO measurements were done without exhalation. Exhaled NO during humming nNO measurements in our study were not corrected for contamination with NO exhaled from the lower airways. We believe that the relatively small concentrations of exhaled NO compared to nNO concentrations during humming can be disregarded. Another variable, potentially affecting nNO humming values, are the amplitude and frequency of the humming. Indeed, exhalation with a fixed flow rate [42] *in vivo* during nasal humming still results in different nNO outputs. To improve reproducibility it should be considered to standardize loudness and pitch of humming.

Recent humming nNO studies are from Maniscalco et al. [40-43]. In one study [41] nNO concentrations in subjects with mild to moderate allergic rhinitis were studied.

Nasal NO measurements were accomplished as described earlier (silent/nasal humming exhalations). The mean nNO concentration in patients during silent nasal exhalation was 32 ppb (= 384 nl/min) and during nasal humming 338 ppb (= 4056 nl/min) with an aspiration flow of 12 L/min. The silent nNO values, obtained with a different method compared to our study, are within normal range (in spite of the allergic rhinitis condition). The humming nNO output however, is much higher compared to our study (4056 nl/min versus 713 nl/min). This probably has to do with the different methods used, or is due to the allergic rhinitis status of the tested subjects. Other model studies [27, 42, 48] have suggested that the NO peak during humming might reflect the patency of the osteomeatal complex. Part of the variability of our data could therefore also be due to between-subject differences in meatal patency.

The usefulness of nNO measurements in diagnosis and monitoring of disease is not yet determined. The present study establishes normal nNO values, which can help in exploring the clinical application of nNO measurements. In addition, measurement of peak nNO during humming may be a method to detect sinus diseases.

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PART 3

NASAL NO AND DISEASE

CHAPTER 3.1

NASAL NO WITH AND WITHOUT HUMMING IN ALLERGIC RHINITIS AND NASAL POLYPS

Submitted

Abstract

Background: nitric oxide (NO) concentrations are high in the upper airways and increase during humming. As upper airway diseases influence nasal NO (nNO), nNO might have capacities as a diagnostic tool. *Objective:* to explore the diagnostic potential of nNO with and without humming in patients with allergic rhinitis (AR) and nasal polyps (NP). *Patients and methods:* nNO was measured in healthy controls (HC) and in patients with AR and NP. Participants held their breath for 10 seconds while air was passively extracted from 1 nostril with 0.7 L/min. Subsequently, they hummed as loud as possible for 10 seconds. The outcomes during breathhold and during humming were recorded. *Results:* 56 adults participated; 23 HC, 22 with AR and 11 with NP. The mean nNO in parts per billion (ppb) during breathhold was 442, 558 and 261 respectively. The median nNO peak (ppb) during humming was 1500 for HC, 1900 for AR and 200 for NP. The difference in nNO without humming between HC and AR was borderline significant (p=0.08). nNO with and without humming in NP was significantly different from the other groups. Humming nNO values seem to discriminate better between NP and HC than silent nNO values. *Conclusion:* nasal NO with and without humming tended to be higher in AR and was significantly reduced in NP compared to HC. Nasal NO might be useful in the diagnostic pathway of nasal polyps.

Introduction

Allergic rhinitis and nasal polyposis are common disorders. The worldwide prevalence is estimated at 21% and 4%, respectively [1, 2]. The diagnosis of these upper airway diseases is based on medical history, physical examination and complementary diagnostic tests, i.e. allergy tests (RAST), skin prick tests, nasal endoscopy and CT-scans. Some of these complementary examinations are expensive, invasive and time-consuming. Hence, there is a need for a noninvasive diagnostic marker and/or monitoring tool in these upper airway diseases.
The concentration of NO in exhaled air is a validated marker of lower airway inflammation [3] In air aspirated from the nose, NO is present in large amounts. Measuring nasal NO (nNO) is easy, not invasive, relatively inexpensive and quick. The reported results on nNO concentrations in upper airway diseases are not consistent [4-13]. This may be due to different methods of nNO measurement, different in- and exclusion criteria or the presence of nasal symptoms at the time of measurement. Recently, it was discovered that humming during nNO measurement resulted in an increased exchange of air between the sinuses and nasal cavity consequently leading to a substantial increase in nNO [14-18]. It is hypothesized that this increase might reflect the patency of the ostiomeatal complex, implicating that nNO measurement with humming might give useful additional information in subjects with possible upper airway disease. The aim of this study was to assess nNO with and without humming in patients with allergic rhinitis and nasal polyps to explore the possible diagnostic value of nNO measurements in these upper airway diseases.

Methods

Subjects

Patients with perennial allergic rhinitis (with or without seasonal rhinitis) and nasal polyps were recruited and selected by means of database search from the outpatient clinics of Otorhinolaryngology and Allergology of the Erasmus Medical Centre Rotterdam. Healthy subjects were recruited through advertisement. In- and exclusion criteria are listed in table 1. All participants were at least 18 years old, signed informed consent and kept a diary on the presence and intensity of nasal symptoms (nasal obstruction, runny nose, sneezing, itchy ear/nose/throat, mucus in nose/throat, headache, loss of smell) 1 week before the nNO measurement. All patients using nasal corticosteroids, antihistamines and/or nasal decongestives discontinued these medications for 6 weeks, 72 hours and 48 hours before the nNO measurements, respectively. Data on potential confounders like gender, age, height, weight, familiar disorders and history of surgery were collected by questionnaire.

Table 1. Inclusion and exclusion criteria of the study protocol.

	Healthy subjects (HS)	**Allergic rhinitis (AR)**	**Nasal polyps (NP)**
Inclusion	- no symptoms of AR / NP - negative SPT - negative endoscopic evaluation for NP	- AR > 1 year - mild to moderate symptoms - positive SPT for at least Drp1 - negative endoscopic evaluation for NP	- symptoms of NP negative SPT -positive endoscopic evaluation for NP
Exclusion	- active smoking - recent surgery in the head and neck area (< 12 months) - recent upper airway infection (< 1 week) - diagnosed asthma - chronic diseases as cystic fibrosis, primary ciliary diskinesia, etc - malignant disease (tumors) - airway influencing medication as corticosteroids, - antihistamines and/or nasal decongestives - not complying with the study protocol		

Abbreviations: SPT=skin prick test, drp1: *Dermatophagoides pteronyssinus*

Skin prick test

Skin prick tests (ALK, Abelló BV, Nieuwegein, the Netherlands) were performed in all patients for 5 common allergens (Dermatophagoides pteronyssinus, cat and dog danders, grass pollen, birch and alternaria). A positive control using histaminedihydrochloride 10 mg/ml and a negative control were carried out at the same time. The skin reaction was measured after 15 minutes and the test was considered positive if the wheal size of the allergen and positive control were 3.0 mm or greater and the wheal size of the negative control was 0 mm.

Examination of the nose

Full diagnostic nasal endoscopy was performed by an ENT-specialist within 8 hours before the nNO measurements. The examiner was kept blinded for the results of the NO measurement. For each side of the nose the investigator evaluated the presence of nasal polyps which had to be at least grade I according to the Lund-Mackay staging system [19].

Nasal NO measurements (silent versus humming)
Nasal NO was measured with a NIOX chemiluminescence analyzer that displayed real-time measurements (Aerocrine, Stockholm, Sweden). Silent nNO values in parts per billion (ppb) were measured during breathhold after a deep inspiration while air was extracted with a flow of 0.7 L/min from one nostril [20, 21]. Subjects were asked to take a deep breath and hold it for 10 seconds. The average nNO concentration at the plateau between 7 and 10 seconds during breathholding was calculated. The maneuver was performed in triplicate. Subsequently the subjects were asked to take a deep breath and start humming (phonation of an 'm') for 10 seconds as loud as possible with their mouth closed. The humming maneuver was performed twice with a 3-minute interval. Because there is no consensus on how to characterize or describe the result of a nNO measurement during humming we recorded different values; the (absolute) peak nNO concentration, the mean of the last 80% of the exhalation (i.e. mean nNO between 2-10 secs) and the concentration at 10 seconds [14, 16, 18]. We defined the presence (yes/no) of an nNO peak as at least 1.5 x the mean nNO during the silent NO measurement [21]. Before each measurement the ambient NO concentration was recorded. The protocol of the study was approved by the Medical Ethical Committee of the Erasmus Medical Centre.

Statistical methods
For the assessment of silent nNO values we calculated the mean of triplicate measurements, and standard deviations. If the outcome showed a normal distribution, we used t-tests to compare between the different groups; otherwise non-parametric tests (Mann-Withney U test) were used. For the analysis of the relationship between nNO and potential confounders (age, gender, extensiveness of nasal polyps, symptom scores) univariate analyses were performed. We also calculated the relative difference between HC and NP for humming and silent outcomes to determine which outcome discriminates best. All analyses were done with SPPS, version 10.1 for Windows.

Results

Study population
Thirty-three adults, of whom 22 with allergic rhinitis (AR) and 11 with nasal polyps (NP), were invited to participate. All patients with NP had a history of ENT intervention versus 50% of the patients with AR. Thirty healthy subjects responded to the advertisement, 23 were included, 7 were excluded due to a positive skin prick test. From the healthy controls (HC) 21.7 % had an ENT intervention (adenotonsillectomy) in the past. See table 2 for the population characteristics.

Silent nasal NO values
All participants were able to perform 3 correct measurements. The mean silent nNO value in ppb (SD) in HC and in patients with AR and NP was 499 (164), 588 (169) and 258 (84) ppb respectively (figure 1). Nasal NO in the group of patients with NP was

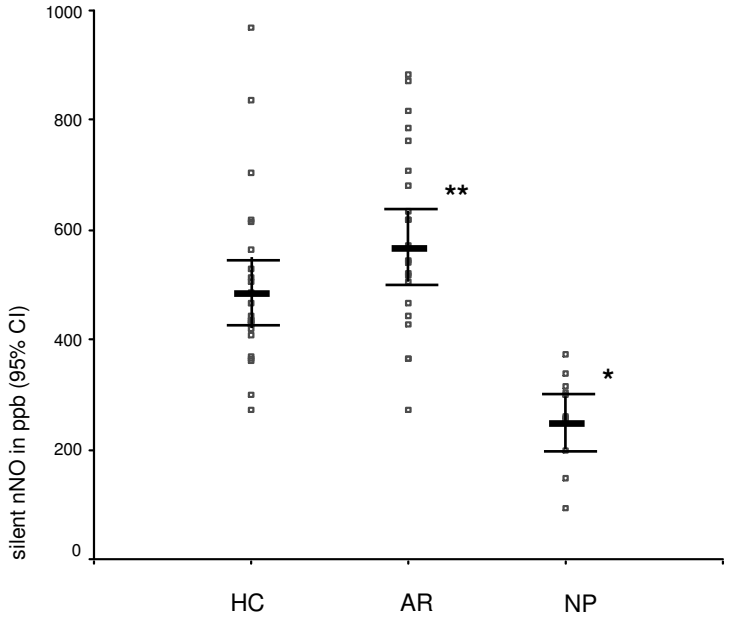
significantly (p<0.01) different from the other groups (HS and AR). The difference between patients with AR compared to HC was borderline significant (p=0.08). The median ambient NO was 2.5 ppb (range:0.0 – 38.2). There was no significant relation between nNO and ambient NO.

Table 2. Group characteristics.

Group	**HC**	**AR**	**NP**
Number (n)	23	22	11
Gender (% women)	68	68	27
Age (SD) (years)	23 (18)	28 (8)	51 (5)
ENT intervention (n)	5	11	11
grommets	-	2	-
ATE	5	9	-
ESS	-	-	11

Abbreviations: HC=healthy controls, AR=allergic rhinitis, NP=nasal polyposis,
ENT=ear, nose and throat, ATE=adenotonsillectomy,
ESS= endoscopic sinus surgery

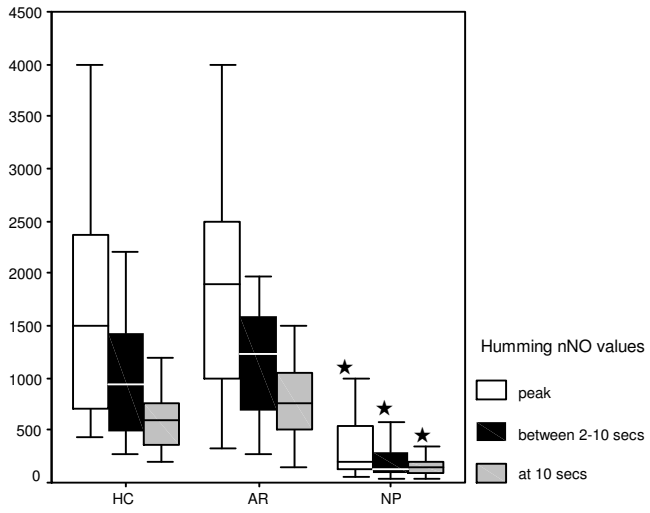
Figure 1. Mean silent nasal NO values (95% CI) in healthy controls (HC), in patients with allergic rhinitis (AR) and nasal polyps (NP). ★= p<0.01 vs. HC, ★★= p =0.08 vs. HC.



Humming nasal NO values

All 65 subjects were able to cooperate with the tests and had no difficulty humming for 10 seconds; however 10 humming measurements (16%) were invalid due to a technical failure. There were no significant differences between the first and the second humming measurements. The absolute peak nNO values (median, range) during humming were 1500 (425-4000), 1900 (320-4000) and 200 (48-1200) ppb, for HC, AR and NP respectively. The nNO humming value during the last 80% of the exhalation were 941 (273-2203), 1230 (273-2971) and 135 (31-642) ppb, for HC, AR and NP respectively. The nNO (median, range) during humming at 10 seconds was 600 (190-2350), 750 (150-1500) and 140 (30-350) ppb, for HC, AR and NP respectively (figure 2). Nasal NO peaks (as defined in 'methods') were present in 84% of the HC, in 82% of the AR patients and in 27 % of the patients with NP (p=0.01). All humming nNO outcomes in the group of patients with NP were significantly ($p \leq 0.01$) different from the other groups (HC and AR) (figure 2). The relative difference between humming nNO in NP versus HC was 0.13, 0.14 and 0.23, respectively for the three humming nNO values while this 0.59 for 'silent' nNO versus healthy controls.

Figure 2. Boxplots showing the median and interquartile range of individual nasal NO values during humming (at the peak, between 2-10 secs and at 10 secs) in healthy controls (HC), patients with allergic rhinitis (AR) and in patients with nasal polyps (NP). ★= $p < 0.01$ vs. HC and AR



Nasal NO values and symptoms (from diary)

There was no significant correlation of reported nasal obstruction, rhinorrhoea, sneezing, itching, headache or mucus and nNO. The nNO value was significantly lower in those reporting loss of smell (401 ppb versus 513 ppb, ($p<0.01$)).

Discussion

In this study we assessed nasal NO (nNO) in patients with allergic rhinitis and nasal polyps. Nasal nNO with and without humming tended to be higher in patients with allergic rhinitis versus healthy controls, but the difference did not reach significance. Nasal NO was significantly lower in patients with nasal polyps than in healthy controls and patients with allergic rhinitis. Humming nNO values seemed to discriminate better between patients with nasal polyps and healthy controls, than silent nNO values.

There are several studies on nNO in rhinitis and nasal polyps [4, 8, 12, 13, 22-28]. An effect of allergic rhinitis on nNO is not consistently reported. However, most studies suggest that nNO is increased in allergic rhinitis [4, 8, 10, 22, 27-30]. We also found higher mean nNO in patients with allergic rhinitis compared to healthy controls but the difference was relatively small (588 versus 499 ppb) and only borderline significant (p=0.08).

Studies on 'silent' nNO in subjects with nasal polyps are limited [5, 16, 23]. In all studies, polyps were associated with lower nNO. Our patients with nasal polyps also had significantly lower nNO compared to healthy controls and allergic rhinitis. The symptom 'loss of smell' was only reported by patients with nasal polyps and therefore negatively associated with nNO concentration (p=0.01).

Humming (phonation of an 'm') during nNO measurement results in an initial increase of nNO (peak) subsequently decreasing to a plateau. Models studies suggested that due to enhanced exchange of air between nasal cavity and paranasal sinuses, which suggests that this measurement could give an indication of the patency of the ostiomeatal complex [14-18]. Humming nNO can be assessed at different time points. We reported the outcome of humming measurement in 4 different ways. The first 3 were the peak nNO value, the mean NO value over the last 80% of the exhalation (between 2-10 seconds) and the NO value at 10 seconds. The second value, mean nNO over the last 80% of the exhalation, has been used in previous studies on this topic [14, 16, 18]. There were no significant differences for all 3 parameters between patients with allergic rhinitis and healthy controls. In contrast, all 3 were lower in patients with nasal polyps. Humming nNO tended to discriminate better than 'silent' nNO (see figure 2) in patients with nasal polyps. The fourth outcome of humming nNO was defined as a peak of at least 1.5 x the mean nNO during silent humming. This outcome showed that nNO peaks were present in 82 % of the controls, allergic rhinitis patients, but only in 27 % of the patients with nasal polyps. This shows that (physical) sinus blockage may indeed influence the humming effect and silent nNO concentration. This was also demonstrated by others [5, 23] who found a

relationship between the extent of nasal polyps and nNO. We did not find this relationship to be significant which might be due to small numbers and also to changed anatomy due to previous ENT surgery, which aimed at improved draignage and hence a larger ostium between sinus and nasal cavity.

The clinical usefulness of nNO remains to be defined, but our results suggest that it can play an additive role in the diagnostic work-up of nasal polyps. 'Silent' nNO measurements seems to give similar information as the combination of 'silent' and humming NO measurements, however humming nNO has a better discriminating capacity in case of nasal polyps.

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CHAPTER 3.2

NASAL NITRIC OXIDE WITH AND WITHOUT HUMMING IN PATIENTS WITH CYSTIC FIBROSIS

Eur J Clin Invest, in revision

Abstract

*Background***:** nasal nitric oxide (nNO) values are reduced in patients with cystic fibrosis (CF). Humming during nNO measurement increases nNO values in healthy subjects and is reduced in patients with CF, sinus disease or nasal polyps. Humming nNO values have not been reported in CF patients yet. We aimed to explore humming nNO values in CF patients and assess whether nNO during humming is a better discriminator than silent nNO measurements in this patient group. *Materials and method*: in a cross sectional study we measured nNO concentrations in healthy controls (HC) and in CF patients, 54 adults participated; 23 HC and 31 with CF. The participants held their breath for 10 s while air was passively extracted from one nostril with 700 ml/min for direct NO measurements (NIOX chemiluminescence analyzer). Subsequently nNO was measured during humming with the mouth closed for 10 s. *Results*: Mean nNO in parts per billion (ppb) (SD) during breath-hold was 499 (164) and 240 (139) respectively. The median nNO peak (ppb, min-max) during humming was 1500 (425-4100) for HC and 120 (23-500) for CF. There was a highly significant difference between nNO both with and without humming between CF and HC ($p<0.01$). The sensitivity and specificity of nNO for detecting CF were better with humming. *Conclusion*: nasal NO concentrations with and without humming are significantly decreased in CF. Humming nNO is an excellent discriminator between HC and CF and performs better than silent nNO.

Introduction

Gaseous nitric oxide (NO) is a highly reactive, lipophilic free radical, which is naturally released in the human respiratory tract. The major part of NO found in exhaled air originates from the nasal cavity and paranasal sinuses. NO is known to act as an aerocrine messenger, a local host defense and as a marker of eosininophilic inflammatory airway diseases [1]. Concentrations of NO in the healthy nose and sinuses (nNO) are high. The nNO concentrations increase 15- to 20-fold while humming compared to a quiet exhalation i.e. silent nNO measurement [2, 3]. It has been postulated that humming nNO reflects the patency of the ostiomeatal complex and therefore could play an additive role in the diagnostic work up or treatment monitoring of airway inflammation [2-4].

Cystic fibrosis (CF) is a serious illness with a worldwide prevalence of more than 0,01%, making it the most common lethal hereditary disease amongst Caucasians [5]. CF is caused by a variety of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CF patients experience recurrent respiratory infections, their airways are colonized with bacteria [6] and the paranasal sinuses are chronically infected and often blocked. Previous research showed that nNO values are low in CF patients. The effect of humming on nNO concentrations in CF patients has not been studied yet. The diagnostic work-up of patients with suspected CF is extensive and nNO may be useful as a marker of sinus pathology.

In this study, we aim to explore nNO concentrations with and without humming in patients with CF and in healthy controls. Subsequently, we assessed whether humming nNO values might increases the discriminative potential of nNO for CF.

Methods

Thirty-one adult patients with CF (mean age 31, SD 9, 12 females) were recruited at random from the outpatient clinic of Pulmonology from the Erasmus Medical Centre Rotterdam. Patients were excluded in case of recent worsening of the disease/ airway infection (< 1 week), doctor diagnosed asthma, diabetes mellitus, pregnancy, active smoking, use of systemic corticosteroids, antihistamines, and nasal decongestives within 3 weeks, 3 days or 8 hours, respectively, before the nNO measurement. On the day of the nNO measurement the nose was inspected by anterior rhinoscopy for nasal polyps. As a control group 23 adult healthy non-smoking volunteers (HC; mean age 23, SD 8 years and 16 females) without any history of allergy or airway disorder took part in the study. Characteristics of the CF patients and HC are presented in table 1. In table 2 we present additional characteristics of the CF patients.

Table 1. Group characteristics

		healthy controls	cystic fibrosis
number	(n)	23	31
gender	(% women)	68	40
age	(years, SD)	23 (18)	23 (8)

Table 2. Characteristics CF patient group (n=31)

Shwachman score* (mean,SD)	64 (9)
Gene-mutation (n)	
classic Δ F508	17
compound	13
missing	1
Colonization type (n)	
P. aeruginosa	13
combinations and/or others	18
ENT intervention** (yes, n)	22
endoscopic sinus surgery	15
adenotonsillectomy	3
grommets	2
other	1
House dust mite allergy (yes)	4

* a score (0-100) to asses the severity of CF based on general activity, physical examination, nutrition and X-ray findings. Excellent 86-100, good 71-85, mild 56-70, Moderate 41-55, severe < 40. ** ENT= Ear Nose Throat.

NO was measured with a NIOX chemiluminescence analyzer that displayed real-time measurements (Aerocrine, Stockholm, Sweden). 'Silent' nNO concentrations (in parts per billion (ppb)) were measured during breathhold after a deep inspiration while air was extracted with a flow of 700 ml/min from one nostril [7]. Subjects were asked to take a deep breath and hold it for 10 s. The average nNO concentration at the plateau between 7 and 10 s after breathholding was calculated. The manoeuvre was performed in triplicate. Subsequently the subjects were asked to take a deep breath and start humming as loud as possible with their mouth closed for 10 s. The peak nNO concentration, the concentration at 10 s and the mean of the last 80% of the exhalation were recorded. The protocol of the study was approved by the Medical Ethical Committee of the University Medical Centre Rotterdam.

Statistical methods

With a statistical power of 80% and a 5% significance level, a sample size of 15 subjects per group was calculated to be able to detect a difference of 750 ppb in humming nNO. At least 20 subjects were planned to recruit to allow for drop outs.

For the assessment of silent nNO we calculated the mean of triplicate measurements, and standard deviations [7]. As humming outcomes we used the peak value (in ppb), the mean value of the last 80% of the exhalation (between 2-10 seconds) and the value at 10 seconds. An nNO peak was defined as at least 1,5 x the average nNO value between 7-10 seconds [7].

If nNO showed a normal distribution, we used t-tests for comparing nNO values between the different groups; otherwise non-parametric tests (Mann-Whitney U tests) were used.

To estimate the discriminative capacity of humming versus silent nNO concentrations in CF we calculated sensitivity and specificity. The cut-off points for sensitivity and specificity were obtained from receiver operating characteristic (ROC) curves.

All analyses were done with SPPS, version 10.1 for Windows.

Results

The mean Shwachman score, a score indicating the severity of CF, in the CF patients was 64, implying that the patients were in reasonably good condition, considering their disease (table 2). There were no nasal polyps found by anterior rhinoscopy in patients with CF.

Figure 1. Distribution of mean nasal NO (in ppb) in patients with cystic fibrosis (CF) and in healthy controls (HC).

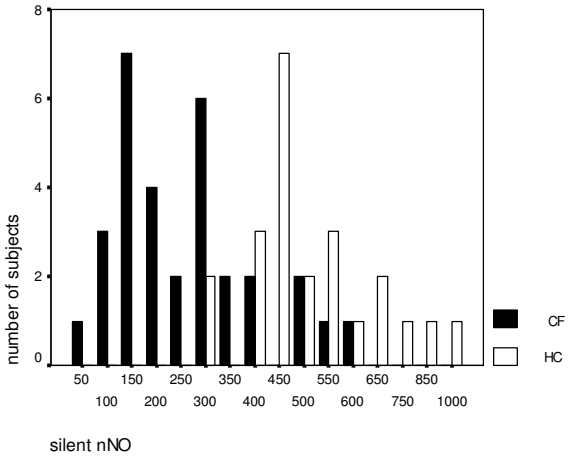
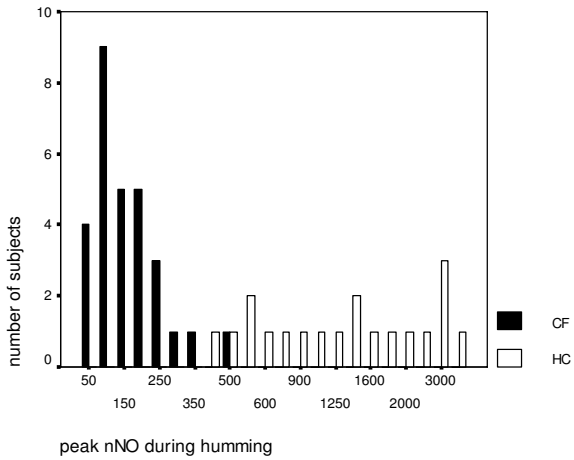


Figure 2. Distribution of peak nasal NO (in ppb) during humming in patients with cystic fibrosis (CF) and in healthy controls (HC).



Nasal NO without humming ('silent' nNO)
The median ambient NO was 2.4 ppb (min 0 ppb -max 13 ppb). There was no significant relation between nNO and ambient NO. All participants were able to perform 3 correct silent measurements. The mean nNO value in ppb (SD) was 499 (164) ppb in HC and 240 (139) ppb in CF patients, ($p<0.01$), (figure 1).

Figure 3. Boxplots showing the median and interquartile range of nasal NO humming concentrations (at the peak, between 2-10 s and at 10 s) in healthy controls (HC) and in patients with cystic fibrosis (CF). ★ $p< 0.01$vs. HC.

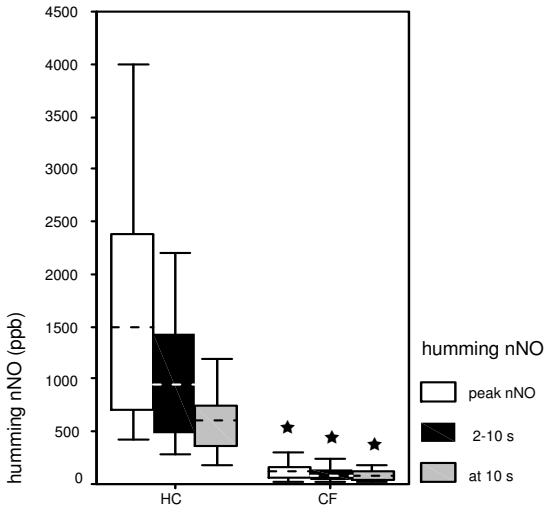


Table 3. Sensitivity (true positive rate) and specificity (true negative rate) at different cut-off points for silent and humming nasal NO.

	cut-off points (ppb)	**sensitivity (%)**	**specificity (%)**
silent nNO	250	100	59
	300	95	72
	350	95	79
	400	79	86
	450	53	90
humming peak nNO	350	100	93
	400	100	97
	450	100	97
	500	95	97
	550	84	100
humming nNO 2-10s	200	100	90
	250	100	93
	300	100	97
	350	95	97
	400	84	97
	450	84	100
humming nNO at 10s	100	100	55
	150	100	79
	200	95	93
	250	90	93
	300	90	100
	350	84	100

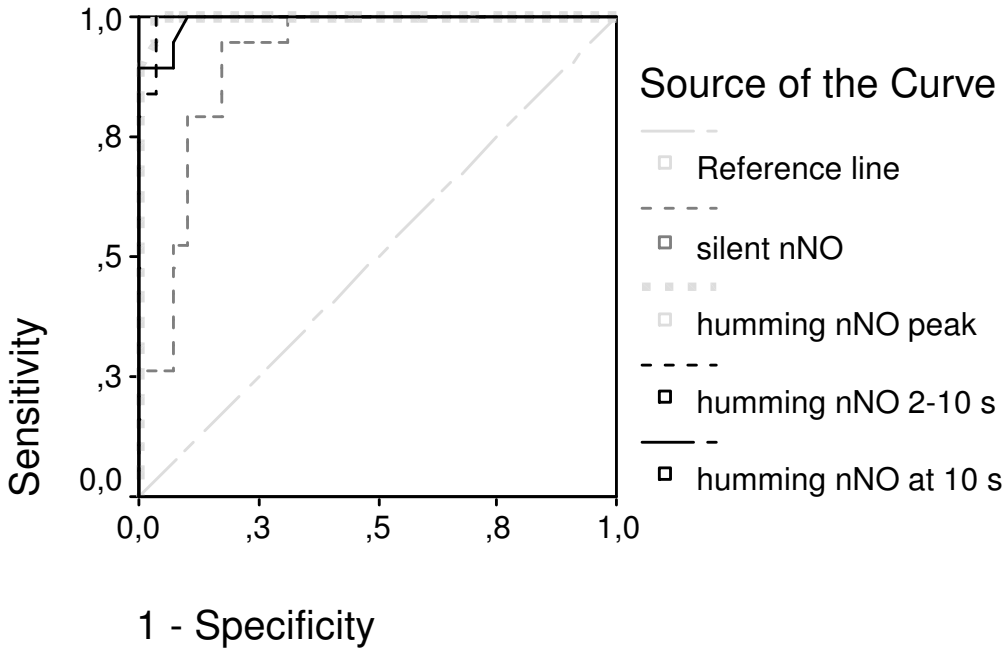
Nasal NO with humming
All subjects except one CF patient were able to perform the humming maneuver and complete the measurements. Due to technical failure 5 measurements (9%) were excluded from the analyses. The median peak nNO value (range) during humming was 1500 (425-4000) ppb in HC and 120 (23-500) ppb in CF patients (figure 2). The median nNO humming value (range) during the last 80% of the exhalation (between 2 and 10 s) was 941 (273-2203) ppb in HC and 78 (16-397) ppb in CF patients. The

median nNO (range) during humming at 10 s was 600 (190-2350) ppb in HC and 85 (14-255) ppb in CF patients. There was a significant difference for all 3 above described humming nNO outcomes in patients with CF compared to HC ($p<0.01$), (figure 3). Seventy-nine percent of the CF patients and 16 % of the healthy controls did not have a peak value (defined as at least 1,5 x the average (silent) nNO value between 7-10 seconds).

Humming versus silent nNO

The sensitivity and specificity for CF of several cut-off points for silent nNO and humming nNO are outlined in table 3. In figure 4 we present ROC curve (receiver operating characteristic) showing the cut-off points with different sensitivity and specificity (any increase in sensitivity will be accompanied by a decrease in specificity) for humming nNO versus silent nNO in patients with CF.

Figure 4. ROC curve representing the trade-off between sensitivity and specificity between silent nasal NO and humming nasal NO (peak, between 2-10 s and at 10 s) concentrations (in ppb). Diagonal segments are produced by ties.



Discussion

We showed that nNO concentrations with and without humming are significantly lower in adult patients with cystic fibrosis (CF) compared to healthy controls. Nasal NO while humming discriminated better between healthy subjects and CF patients compared to silent nNO.

In literature nNO values are consistently reported to be lower in CF compared to healthy subjects [5, 8-10]. In accordance we found significantly lower 'silent' nNO values in patients with CF, compared to healthy controls. Low nNO levels in CF seem paradoxical since neutrophilic airway inflammation, as in CF, leads to release of cytokines which are known to upregulate iNOS and thus increased NO output. The reduced nNO in CF is probably the result of several factors including a defect in the inducible nitric oxide synthethase (iNOS) gene expression [11], impaired diffusion and increased metabolization of NO [10, 12, 13], destroyed epithelial cells [14] and/or reduced ciliary function [15]. It was postulated that there is a close linkage between the CF gene and the iNOS gene with coinheritance of defects in both genes. A similar mechanism has been shown in patients with primary ciliary dyskinesia [10, 11] but not in CF.
Humming while measuring nNO increases nNO in healthy subjects. In subjects with obstructed sinuses and/or nasal polyps, humming nNO values are relatively low [3, 16]. Hence, nNO is suggested as an indicator of the patency of the ostiomeatal complex [17]. In CF patients humming nNO values have not been reported. We found that humming nNO values in CF are much lower than in healthy controls. In 96% of the patients with CF there was no peak . Moreover: nNO has a better ability than silent nNO to correctly classify those with and without CF. The type of humming outcome (i.e. peak nNO, nNO 2-10 s and nNO at 10 s) is not essential, as all 3 outcomes show higher sensitivity and specificity for CF compared to silent nNO values.

The low nNO humming values could be due to obstruction of the ostiomeatal complex as a result of mucous plugs or as a consequence of nasal polyps. One third of patients with CF have nasal polyposis [18]. In our study nasal polyps were not detected by rhinoscopia anterior but this does not exclude the presence of nasal polyps in the sinuses. Hence we hypothesize that obstructed sinuses by mucus or polyps can explain the low nNO concentrations in our CF patients. In addition, similar to 'silent' nNO concentrations a combination of a defect in the iNOS gene expression [11], increased metabolization of NO [10, 12], damaged epithelial cells [14] , reduced ciliary function [15] and/or thick mucous) may contribute to a low humming nNO.

In conclusion, we showed that nNO concentrations with and without humming are significantly lower in patients with CF compared to healthy controls. Furthermore humming nNO discriminated better than silent nNO between healthy controls and CF patients. We propose that humming nNO measurement is a useful additional test in the diagnostic work-up for CF.

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PART 4

GENERAL DISCUSSION

Introduction

This chapter will address and discuss aspects of the measurement of nasal NO (nNO), its normal values, outcomes in upper airway disease, diagnostic potential and the implications of our results for further research in nNO.

Nasal NO measurement

The upper respiratory tract is a complex system of communicating cavities consisting of the paranasal sinuses, the Eustachian tube, the middle ear and the pharynx down to the larynx. Each of these cavities may contribute to the NO concentration measured in the nose. For this study we arbitrarily studied this tract only superiorly of the palate. The measured nNO concentration depends on both the amount of NO produced in the upper airways (in a given time) and on the amount of nNO which is absorbed, metabolized or removed [1]. To measure nNO generation of airflow through the nasal cavity (transnasal airflow) is required. This flow can be achieved with the nostrils in series, by means of aspirating or insufflating air via one nostril, while the velum is closed. It can also be achieved with the nostrils in parallel, by means of exhaling via one or both nostrils while aspirating via the mouth which can be done with air entrained into both nostrils during breath hold, or by aspirating from one or both nostrils with the mouth open during breath hold [2]. Whatever method used, it is essential to know the velocity of the generated transnasal airflow because nNO concentrations are flow dependent [1]. In general it is recommended [3, 4] to measure nNO through the nasal cavities in series by aspirating air via one nostril with a constant flow while the velum of the palate is closed. The product of transnasal air flow and measured nNO concentration permits calculation of nNO output (nNO output (nL/min) = flow (L/min) x nNO (ppb)) and also allows us to more carefully compare nNO studies using different transnasal flows [1, 5]. Present evidence suggests however, that nNO output is only relatively constant over a range of flow rates between 1 and 5 L/min [5, 6]. At transnasal flow rates < 0.3 L/min, NO may be absorbed by nasal mucosa, reducing the calculated nNO output. It is therefore surprising that most studies on nNO use transnasal air flows < 1L/min or even smaller than 0.3 L/min [3, 7-20].

In this thesis we used the measurement method as recommended by the American Thoracic Society [4] with sampling from one nostril while the velum is closed and examined the effect of three different transnasal air flows (chapter 2.1). Nasal NO was measured during ten seconds. Our experiments showed a characteristic nNO flow curve with a constant increase of nNO until a certain – steady state – plateau value, with all three sample flows. With an airflow of 0.28 L/min the steady state NO plateau phase was not always reached within ten seconds. The highest transnasal airflow gave an unpleasant feeling in the nose resulting in reduced acceptance by subjects, especially in children, consequently increasing failure rates. The nNO concentrations

were not influenced by the nasal cycle and time of measurement, the measurements had a good reproducibility. Eventually we choose, from a clinical perspective, the 0.70 L/min transnasal airflow in our further research, as this flow was well accepted, feasible and resulted in reproducible results.

In 2002, during a regular experiment on nNO in healthy volunteers Weitzberg et al. [21] accidentally discovered that 'humming' – phonation of an 'm' or 'n'– during the nNO measurement results in a different flow curve compared to characteristic nNO flow curve. Instead of a relatively slow increase of nNO towards a plateau they found an instant, rapid, increase of nNO to a peak value, followed by a quick decline to a plateau value. The nNO increase as seen during humming is postulated to be due to increased washout of NO accumulated in the sinuses [21, 22]. Peak and total nNO decreases after repeated consecutive humming but a complete recovery of the peak is observed after, at least, three minutes of normal breathing. Weitzberg suggested that humming might give information about the relative contribution of NO from the nose and the sinuses, as well as on the patency of the sinus ostia [21, 22].
In their humming research, Lundberg et al. and Maniscalco et al. [22-25] used a tight-fitting mask covering the nose for nasal measurements and a mouthpiece for oral exhalations. The subjects started each manoeuvre by inhaling NO free air through the nose and then exhaled at a fixed flow rate (12 L/min) either quietly or with nasal humming. The time of exhalation varied and loudness of humming was not described. Loudness of humming influences the humming values, the louder the humming the higher the peaks. This is probably due to the increased amplitude of the phonated consonant ('m'). The pitch of humming does not evidently influence the humming values. This is not studied systematically, though, we found, in a preliminary study, that humming values are not different in women versus men.
In this thesis we used another method to measure nNO during humming compared to Lundberg et al. and Maniscalco et al. We measured nNO during humming while sampling from an olive placed against one nostril, afterwards subjects were asked to inhale as deep as possible and start humming (nasal exhalation) as loud as possible for ten seconds. Meanwhile nasal air was extracted from the nose with a transnasal flow of $\geq$ 0.70 L/min (chapter 2.3, 3.1 and 3.2). The nNO curve produced on-line is comparable to the nNO curve as produced with the method used by others [21-26].

Nasal NO measurement – practical problems

There are hardly any reports on practical problems in nNO measurements. This is remarkable in view of obvious difficulties when measuring nNO, especially in cases of nasal obstruction as for instance in rhinitis, sinusitis and/or nasal polyps. After all, when there is a certain amount of retained mucous and/or mucosal swelling in the nose, leading to obstruction, measurement of nNO with the aspiration technique will result in a vacuum and subsequently impaired NO measurements.

In our study on nNO values in children, we had problems in obtaining valid NO concentrations in 15 % of the participants. Part of these problems were (probably) age related, namely difficulties in maintaining an adequate palatal closure, failure to achieve a stable plateau, inability to hold breath for ten seconds or not understanding the measurement procedure. In some children, the measurements could not be accomplished due to nasal congestion and/or mucous plugs, consequently resulting in absence of flow and inability of measuring NO. Blowing the nose helped in some subjects, but not in all. We did not encounter these problems in adults, which might be due to chance or to their different anatomy (larger nasal passage).

Nasal NO normal values

NO is generated from arginine by a family of NOS enzymes. Three distinct isoforms of human NOS have been characterized to date. Two of these isoforms, endothelial and neuronal NOS, are constitutively expressed and changes in their activity regulate vascular tone, platelet activation and neurotransmission. The third isoform has been found to be expressed upon stimulation with pro-inflammatory cytokines by most cell types within the organism and has been called inducible NOS (iNOS). It is showed that epithelial cells lining the sinuses of healthy subjects express a NOS characterized as the inducible isoform. This type of NOS is mainly responsible for the high sinus and nNO concentration produced in the normal upper airways [27-30].
To explore the diagnostic usefulness of nNO measurements in clinical practice normal values need to be established. Studies on nNO normal values are limited. There is one study by Daya et al. [12] that formally intended to determine normal values in children and one study in adults by Bartley at al [31].
Daya et al. [12] found a mean nNO output of 480 nl/min, the mean nNO output in healthy children in our normal value study was 312 nl/min. The difference is most certainly due to the different aspiration flows used; 3.0 L/min in Daya's study versus 0.7 L/min in our study. It might also have to do with the fact that Daya did not correct for potential confounders. We found a positive association between nNO and age but also between nNO and history of an adenoidectomy and ambient NO (chapter 2.2), while Daya did not find an association with age and did not report an association with history of an adenoidectomy and ambient NO.
The association between nNO and age in children less than 12 years old was approximately three times stronger compared to children aged 12 years and over. In the latter group the association was not significant anymore. We found a significant negative association between history of adenoidectomy and nNO but only in children < 12 years. So, measuring nNO after an adenoidectomy results in significantly lower nNO concentrations. We speculate that this only occurs in children < 12 years old because the adenoid regresses rapidly after this age [32, 33], making children with a history of an adenoidectomy comparable to children without. Besides, removal of the adenoid (which relatively increases the nasal airway volume, resulting in lower nNO) is more likely in case of chronic respiratory infections (which potentially increases

nNO) [20, 34, 35]. We hypothesize that an adenoidectomy results in a decrease of nNO as the cause of the infection is removed and the volume of the upper airway increases.
Ambient NO showed to be related to nNO as well. Our study was conducted in two primary schools, in an urban area situated close to busy motorways. The NO measurements were mainly performed during traffic rush hours, inducing higher levels of ambient NO due to pollution. This is reflected by the high ambient NO we measured (chapter 2.2). High environmental NO can induce a problem in separating a real nNO change from ambient NO change when nNO is intended to be used as a diagnostic tool or monitoring tool. For example, monitoring allergic rhinitis where there might be only small changes in nNO. Several authors [14, 31, 36-41] monitored ambient NO during every nNO measurement but did not report the values or any correlation with nNO [8, 42-45]. Others subtracted ambient NO from nNO [14, 46] but without any good arguments. In chapter 2.2.we showed that correction for nNO had to be made by subtracting 0.50 per ppb of ambient NO (chapter 2.2). In our studies on nNO in adults an association between nNO and ambient nNO was not found. In these studies ambient nNO, however, did rarely exceed 20 ppb, and this is probably the case in many studies. Consequently, one can assume that ambient NO of less than 20 ppb does not affect nNO. We recommend that ambient NO values should be monitored and reported and if necessary corrections should be made for instance as ambient nNO > 20 ppb.

Bartley et al. [31] obtained normal nNO values in European non-smoking healthy adult volunteers. They found a lower mean nNO output (218 nL/min) compared to our study on normal values in adults (319 nL/min) (**chapter 2.2**). The difference between our studies and Bartley's study might be explained by difference in used equipment, a different duration of breath-hold (not reported in Bartley's study) and by another definition of the plateau value. Also the lower flow used by Bartley et al. might have potentially resulted in relatively more absorption of nNO by the nasal mucosa, and thus lower nNO output.

Summarizing, we established normal values for children from six years upwards and in adults. We do emphasize that these normal values can only be applied when using the same measurement technique as presented in this thesis. Nasal NO in children younger than 12 years are not necessarily comparable with nNO in older subjects and ambient NO should be monitored. Incorporating healthy control groups in studies on nNO in disease should be considered depending on the research question.

Normal nNO values for humming were not available. There are five studies [21-25], by one research group, on nNO and humming. Of these, four describe humming nNO values in healthy volunteers [21-23, 25]. Their reported humming outcomes differ considerable per study (see also chapter 2.3). This has probably to do with differences in time of humming i.e. the exhalation (5 versus 10 seconds) or transnasal airflow

used (6 L/min versus 12 L/min). The differences may also be due to different definitions of the outcome (mean nNO output over the last 70 or 80% of the exhalation) and the small research groups (n=10). In our study on humming in healthy subjects (n= 40) (**chapter 2.3**) we found, like reported by others [21-25], an initial increase towards a peak value in nNO during humming compared to a measurement without humming.

Assessment of humming nNO in children has not been reported yet. We explored humming nNO values in a few children (< 11 years) but peak nNO values were mostly absent (unreported data). We could hypothesize that this has to do with the development of the sinuses, as children aged 12 years and over did produce nNO peaks during humming, but this should be further explored.

In conclusion, despite methodological differences, all studies on humming until now, found that humming results in an initial and average increase of nNO compared to silent nNO measurements. The next step should be to find out whether humming could indeed detect impaired ostiomeatal complex patency and whether humming nNO values can act as an objective measure for this patency. We will discuss these possibilities in hereafter.

Nasal NO and disease

Allergic rhinitis is a manifestation of the atopic syndrome. Tissue eosinophilia is a characteristic feature of the late response phase of the allergic reaction. The eosinophil is a source of leukotrines, prostaglandins, platelet activating factor, cytokines and cytotoxic proteins. As a response to the released inflammatory mediators iNOS expression is increased resulting in increased NO production. This increase in NO is, accordingly, measured in the exhaled air (FENO) [47]. Hence, FENO is a marker of eosinophilic inflammation in the bronchial tree. In patients with allergic rhinitis, which involves an eosinophilic inflammation as well, nNO is generally not significantly elevated. This is striking and seems contradictive, as the expression level of different NOS forms, iNOS in particular, was shown to be high in the nasal epithelial tissues of patients with allergic rhinitis [28, 29, 48-51]. The rise in iNOS activity mainly found in inflammatory cells, epithelial cells, endothelial cells and in glandular elements of the allergic nose, is probably due to persistent exposure and/or mucosal inflammation. In normal controls submucosal infiltration of iNOS is not observed in general [29].
The fact that the majority of studies on nNO and allergy found a trend towards increased nNO (see chapter 1.2), independent of measurement method can (partly) be explained by several hypotheses. While NO diffuses from the submucosal area towards the nasal cavity, NO can interact with other biological substances as, for instance, oxygen and hemoproteins. This interaction might result in an insignificant increase of measured nNO output in allergic rhinitis. Another hypothesis could be the

diffusion of NO through the ostiomeatal complex. The patency of this complex changes continuously, hereby influencing the amounts of nNO in the nasal cavity. The high background levels of NO in the nose could easily blunt subtle changes in NO production in the order of magnitude as seen in the lower airways as well.
Of interest are the findings of Arnal et al. [37] and the findings we did in chapter 3.2. Arnal et al. [37] found increased nNO concentrations in allergic patients with nasal polyps versus controls and versus patients with nonallergic nasal polyposis. In addition, we found that allergic patients with cystic fibrosis (CF) had higher nNO compared to CF patients without an allergy. The numbers are quite small but we see that allergy, in diseased upper airways (i.e. nasal polyps [15] or CF) in which low nNO is common (see chapter 3.1 and 3.2), results in a substantial increase of nNO. This might suggest that nNO in allergy is derived from a type of NOS that resembles iNOS but the expression and/or activity could be different [20, 35, 37, 52]. This hypothesis is supported by Lundberg et al. [52]. Systemic steroids are well-known inhibitors of iNOS (type II NOS) [53] but Lundberg et al. [52] showed that nNO concentrations in volunteers with normal upper airways are not influenced by high systemic doses of corticosteroids [52]. The ability of topically applied steroids to decrease both nasal symptoms and nNO levels suggest that the elevation of nNO in allergic rhinitis is unlikely to be derived from paranasal sinuses, which should not be affected by steroids applied in nasal sprays [20]. Another hypothesis, showing that nNO in allergy could well be derived from a type NOS resembling iNOS, is a defect in the iNOS expression in CF patients resulting in low nNO.

Until now the present data shows that measurement of nNO does not have an additional contribution in the diagnostic pathway of allergic rhinitis. We do however, think that nNO in patients with allergic rhinitis should be further explored as a monitor of treatment effects as changes in nNO in a single subject can well be monitored by nasal nNO [14].

Nasal polyps are benign growths of the nasal and sinus mucosa, mainly situated in the middle meatus [54, 55]. Their preferred site of origin is the mucous membrane of the outlet of sinuses. Polyps can occur in chronic sinusitis, allergic rhinitis and cystic fibrosis. The pathogenesis of nasal polyps is not known. Most theories consider polyps to be a manifestation of chronic inflammation. Histological, nasal polyps are composed of a loosely organized mucoid stroma and mucous glands and are covered by respiratory epithelium, which often exhibits foci of squamous metaplasia. Prominent thickening of the basal membrane is a common finding [56]. The polyps are infiltrated by lymphocytes, plasma cells, mast cells, neutrophils and eosinophils. Eosinophils are a prominent and characteristic feature in 80-90% of the nasal polyps, whereas polyps with lymfocytes and neutrophils as predominant cells occur in CF and primary ciliary dyskinesia (PCD). How eosinophilic inflammation leads to polyp formation remains unclear. Immunohistochemical studies on polyps show that the total NOS, mainly existing out of iNOS activity, is higher than in the nasal mucosa

(with only cNOS activity). In both, NOS is localized in the epithelial cells. Two studies on nNO in patients with polyps show low nNO values compared to healthy controls as well as a direct correlation between the extent of the polyps and nNO [15, 37]. We also found that patients with nasal polyps had significantly lower nNO compared to healthy controls and patients with allergic rhinitis (chapter 3.1). The low nNO levels may well reflect the ostiomeatal obstruction. However, this does not explain the correlation between extent of polyps and nNO [15, 37]. Small polyps can already block the ostiomeatal complex and extension thereafter takes place towards the anterior of the nasal cavity. It also does not explain, why Arnal et al. [37], found similar or even higher nNO levels in allergic patients with nasal polyps compared to healthy controls. They found, as expected and in accordance with literature, low nNO values in patients with nasal polyps alone. The finding of Arnal et al. might be due to chance as the group of patients with nasal allergy and polyps was small (n=7), but it makes the sinuses as the main origin of nNO in, at least, allergic rhinitis, questionable. It shows that low nNO probably is not explained by ostiomeatal obstruction alone. Is there another factor increasing nNO in case of nasal polyps combined with allergy? Allergic polyps do have an increased number of eosinophils compared to non-allergic polyps. The eosinophils are recruited by eotaxin and eotaxin subsequently increases nNO [51]. At its turn NO is toxic in high concentrations and might therefore lead to epithelial damage, which is needed for the formation of polyps [56-58]. Besides, the NOS activity, mainly iNOS, is high in case of allergy (see chapter 1.2 and first section of paragraph 4.5) so it may be possible that both factors (increased iNOS activity and increased nNO due to eotaxin) result in high nNO levels in the nasal cavity despite the ostiomeatal occlusion. It can also be hypothesized that NO in allergy is derived from an alternative type of iNOS (see previous paragraph).
The books are not yet closed on the cause of the higher nNO in allergic nasal polyps. Certainly, confirmation of the study of Arnal et al. is needed. We are optimistic about the diagnostic capacities of nNO in nasal polyps and it might well find a place in the diagnostic work-up of patients with this disease.
Cystic fibrosis (CF) is a chronic multisystem disorder characterized by recurrent endobronchial infections, progressive obstructive pulmonary disease, and pancreatic insufficiency with intestinal malabsorption. CF is caused by a variety of mutations in the CF transmembrane regulator (CFTR) gene on chromosome 7. In literature nNO is consistently reported as reduced in CF patients [59-62]. A defect in the iNOS expression [61, 63] could explain this. It is also postulated that there is a linkage between the type of CFTR gene mutation and the gene expression for iNOS with coinheritance of defects in both, as is the case in patients with primary ciliary dykinesia [63]. Other factors that are suggested to contribute to low nNO values include reduced ciliary function, thick mucus lining, destroyed epithelial cells and increased degeneration of nNO. The latter has been observed by several authors who found nNO metabolites as nitrite and nitrate to be increased in the CF sputum [1, 18, 61, 64]. In our study on nNO and CF (chapter 3.2) we found that nNO values were approximately 50 % reduced compared to the nNO values in healthy adults. The nNO

values in our study were independent of type of colonisation, type of gene mutation or severity of CF. One study on nNO and colonisation did find lower nNO in patients colonized with Pseudonomas aeruginosa compared to non-colonized patients [65]. Thomas et al [18], however did not find this correlation and they also did not find a correlation between nNO and genotype. In chapter 3.2 of this thesis, all patients were infected, and there was no difference between patients infected with Peudonomas aeruginosa and other types of colonisation. We examined our CF patients' noses by anterior rhinoscopy. Nasal polyps were not observed, which does not exclude nasal polyps but large obstructing polyps were not present. So theoretically, nasal polyps i.e. meatal obstruction (probably) could have resulted in low nNO values. In conclusion, nNO measurement may be useful in the diagnostic pathway of CF. If nNO is low, CF should be considered.

Humming nasal NO and disease

Humming, as described earlier, modifies (on-line) nNO curves. In healthy subjects it results in an increase of (peak) nNO. Five studies on this subject have been conducted of which two on healthy subjects, two on patients with (allergic) nasal polyps or allergic rhinitis and healthy controls and one study on allergic patients only. These studies all aimed to compare the nNO values with and without humming in the same patient. We, on the other hand, aimed to compare nNO values with and without humming in healthy subjects with subjects with an upper airway disease, to assess whether humming may have an additive value to nNO measurement without humming.
In our study on humming nNO in allergic patients (chapter 3.1) the nNO outcomes tended to be increased in patients with allergic rhinitis compared to humming nNO outcomes in healthy controls. This also goes for nNO values without humming; meaning that humming does not seem to have an additive role compared to nNO without humming. Is this true? If humming nNO reflects meatal patency it would be potentially useful. One study on humming nNO and allergic rhinitis by Maniscalco et al. [24] aimed to assess whether peak nNO values could be used to detect sinus abnormalities in 59 patients with untreated mild to moderate allergic rhinitis. They concluded that absence of peak nNO during humming was associated with endoscopic findings suggestive of sinus ostial obstruction in subjects with allergic rhinitis. As in many studies there is a definition problem since one could question the definition of 'sinus abnormality' but also the definition of humming nNO outcome. In Mansicalco's study humming nNO outcome was defined as humming nNO minus the nNO value without humming. Subsequently, the resulting value had to be above 3 standard deviations from the mean nNO levels without humming, to be called a peak. This outcome definition resulted in an absence of peaks in 80% of the cases. It is unfortunate that there was no control group in this study. Healthy controls could also have lacked nNO peaks (during humming) with this outcome definition. The fact that definition of outcome is essential is demonstrated by the same investigators [25]. In

another study on nNO in allergic patients, they found increased humming nNO values. But here another definition outcome for humming nNO was used (mean nNO output over last 80% of the exhalation). We used and compared different humming nNO outcomes too. In chapter 2.3 we assessed the peak nNO value during humming, the plateau value (mean nNO between 7 and 10 seconds) and the presence of a peak (yes-no). In chapter 3.1 and 3.2 humming nNO outcomes were defined as peak nNO and as the presence of a peak as well but also as the mean value over the last 80% of the exhalation and as the value at 10 seconds. We did not investigate which outcome measurement is best. In essence there are probably no big differences between the different humming nNO outcomes. After all we showed, in part 3 of this thesis that all humming nNO values appeared to be significant or non-significant compared to silent nNO values. We do however, tend to prefer the outcomes based on a mean over time, as the peak values and the value at 10 seconds are more prone to instability and assessment of the presence of a peak is more arbitrary.

We showed in chapter 3.1 that humming nNO outcomes in patients with nasal polyps are significantly decreased compared to healthy controls and patients with allergic rhinitis, as are the mean nNO values without humming. However, the relative difference in nNO values with and without humming is larger in humming. Patients with nasal polyps had low silent nNO values but even lower humming nNO values compared to healthy controls (chapter 3.1). This might mean that humming has a better discriminating power to separate between normal nNO and nNO in case of nasal polyps. It could also be speculated that humming is more useful in case of patients with allergic rhinitis and nasal polyps [37]. NO values without humming are in this respect confusing but humming nNO might perform better.
In future research on nNO and humming, it would be interesting to comprise assessment of nNO in patients with nasal polyps with and without allergy and humming. Another suggestion for prospective research is assessment of the discriminating capacity of nNO (with or without humming) in patients with nasal polyps with- or without allergy.
Humming nNO values in patients with CF are not previously reported. We found low or absent nNO peaks compared to healthy controls. The type of humming outcome (i.e. peak nNO, nNO 2-10 s and nNO at 10 s) is not essential, as all three outcomes show higher sensitivity and specificity for CF compared to silent nNO values. In addition, there were no peaks (defined as at least 1,5 times the average (silent) nNO (ppb) between 7-10 seconds) found in 96 % of the patients with CF. In contrast peaks were present in 84% of the healthy controls (chapter 3.2). These findings imply that CF is very unlikely in the presence of peak nNO.

Conclusions and considerations for future research

This thesis aimed to explore whether nNO can be used as a diagnostic tool in clinical practice. First we examined the methodology of the most frequently used nNO

measurement method. After defining the methodology for clinical practice, normal nNO values were established in children and adults. The nNO measurement with and without humming showed to be easy, quick and feasible. Thereafter we explored some diagnostic potential of nNO in allergic rhinitis, nasal polyps and CF.
The diagnostic properties of nNO in case of allergic rhinitis are limited. More extensive knowledge on the site(s) of origin of nNO is needed to get more insight in the different reports on nNO concentrations in allergic rhinitis. Nasal NO, however could still play a role as a monitor of medication therapy in this disease.
In case of nasal polyps and CF nNO can very well play a role in the diagnostic pathway as an additional test. Humming nNO can help to discriminate more clearly between healthy subjects and nasal polyps or CF. In case of low nNO one should be aware of these diseases and use additional tests to discriminate.
Finally, we can say that there is still a lot to be learned on nNO, but the present knowledge suggests that it has the potential to become a useful tool in diagnostic pathway of nasal polyps and CF.

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PART 5

SUMMARY, SAMENVATTING

Summary

Nitric oxide (NO) is a gas with which is found in high concentrations in the upper airways. In the lower airways the concentrations are a hundred fold lower. It is not quite clear why NO concentrations in the nose and paranasal sinuses are higher. Scientists hypothesized that the high concentrations of the gas may provide for the sterility of the paranasal cavities but it could also play a role in the activity of the ciliary function of the mucosa. In addition it is suggested that nasal NO (nNO) functions as an aerocrine mediator involved in the regulation of the ventilatory function.
The concentration of NO in the lungs – i.e. lower airways – is validated as a marker of eosinophilic inflammation and is also utilized to monitor treatment therapy. For instance, the NO concentrations in lungs of asthmatics are increased but decrease while treating with inhalation steroids. Whether NO concentrations in the nose can be used as a marker of inflammation of upper airway diseases as for instance allergic rhinitis, nasal polyps or more uncommon, cystic fibrosis (CF), is not evident yet. This is because, among other things, lack of consensus on measurement methodology of nNO subsequently leading to variable study results. Measurement of nNO in general shows a slow increase towards a stable steady state plateau. In addition, one research group discovered only recently that phonation of an 'm' or 'n' ('humming') while measuring nNO, results in modification of the characteristic nNO curve. Instead of the slow increase there is an instant rapid increase to a peak value followed by a quick decline to a plateau value. Scientists postulate that the peak value, which is absent during a silent measurement, might give an indication of the patency of the paranasal sinuses to the nasal cavity (ostiomeatal complex). Hence, humming might add valuable information to nNO measurements with regards to the situation of the ostiomeatal patency. The clinical relevance of a nNO measurement while humming is however, not known yet.

In this thesis we determined how nNO is best measured; subsequently we established nNO normal values in children and adults. We examined the effect of humming on nNO in healthy subjects and subsequently we assessed whether nNO measurements with and without humming can contribute in the diagnostic pathway of the upper airway diseases allergic rhinitis, nasal polyps and CF.

In **chapter 1.2** we reviewed literature on the origin, production and measurement of nNO as well as the effect of allergic rhinitis, nasal provocation and medication on the NO concentration in the nose. We conclude that there is consensus on the measurement method of nNO is missing. The expression of the NO-synthase (NOS), an enzyme inducing NO production, is increased in allergic rhinitis and that the season apparently does not influence nNO. The origin of nNO appears to be mainly situated in the paranasal sinus, although discussion remains regarding the exact source of nNO.

In **chapter 2.1** we take a closer look at the measurement of nNO. We chose a method which is recommended by the American Thoracic Society, namely measurement of nNO during breath hold. An NO inert olive is placed against 1 nostril, hereafter the volunteer was asked to take a deep breath and hold it for 10 seconds. Meanwhile air is actively extracted from the nose (transnasal airflow). The amount of nNO is directly measured and presented on-line. The extraction velocity i.e. aspiration velocity is important because nNO is flow dependent i.e. a higher aspiration flow gives lower nNO concentrations. A well performed measurement is characterized by a curve showing a continuous increase in the NO concentration until a stable plateau value is reached. The measured nNO concentration is the mean of 3 measurements, i.e. mean nNO between 7 and 10 seconds. With this method we explored the effect of 3 different transnasal airflows (0.28, 0.7 and 1.2 L/min), the nasal cycle, time of the day and the reproducibility on nNO concentrations. In concordance with literature we also found that nNO concentrations are dependent on the aspiration velocity. Time of the day and nasal cycle did not influence the measurements and with an aspiration velocity of 0.7 L/min the nNO measurement is quick, reproducible and feasible.

In the next two **chapters, 2.2 and 2.3**, nNO normal values are established in 340 children and in 100 adults. The mean nNO value in children is 449 ppb (parts per billion). In children younger than 12 years, the nNO value is associated with age and a history of adenotomy. In all children (> 18 years) nNO is influenced by ambient nNO. The mean nNO in adults (> 18 years) is 455 ppb, which is independent of age and ambient NO. NO concentrations in the nose are independent of gender, passive smoking and body mass index for all ages. In **chapter 2.3** nNO is also assessed while humming. From the 100 adults 40 were randomly picked and asked to phonate an 'm' as loud as possible during the nNO measurement twice with a 1 minute interval. The median peak value was 1019 ppb the first time and 837 ppb after the second measurement. The difference is significant and is probably the effect of the temporary shortage of NO from the paranasal sinus. The mean plateau value (after the peak) during humming appears to be equal to the mean plateau value during the silent measurement.

Chapter 3.1. describes the assessment of nNO concentrations with and without humming in healthy adults and in patients with allergic rhinitis and nasal polyps. The mean nNO in patients with allergic rhinitis tended to be increased compared to healthy controls but was only borderline significant. The mean nNO is significantly lower in patients with nasal polyps compared to healthy controls. The median humming nNO values in patients with allergic rhinitis were comparable to the nNO values in healthy controls. In patients with nasal polyps, on the other hand, humming nNO values were significantly lower compared to healthy controls. It also seems that humming has an additive value to nNO measurements as humming nNO values discriminate better between healthy controls and nasal polyps than silent nNO values.

In **chapter 3.2** we looked at nNO with and without humming in patients with cystic fibrosis (CF). CF is caused by a variety of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Patients with CF experience chronic respiratory infections and their airways are colonized with bacteria. It is known that the nNO values in CF are low but humming nNO values were not described yet. We found, as in the literature on nNO and CF, significantly lower silent nNO concentrations in the CF patients in comparison to healthy controls. The humming nNO values were significantly lower too and here, humming showed to discriminate better between healthy controls and cystic fibrosis than silent nNO values as well.

In the 4th part of this thesis we discuss our methodology, our results and we present suggestions for future research on nNO. This thesis aimed to assess whether nNO has a diagnostic capacity in upper airway diseases. The measurement itself is simple, quick, and easy applicable in clinical practice. In our opinion it is not yet evident whether nNO, with and without humming, can be used as a diagnostic tool in allergic rhinitis. In contrast, we think that measurement of nNO could be of diagnostic value in nasal polyps and cystic fibrosis, at least as a screening tool especially in combination with humming. With the remark that nNO values, with and without humming, in patients with nasal polyps and in patients with CF are similar (low). In the future nNO could be used as a screening tool for these diseases and perhaps also in allergic rhinitis. Nasal NO might also be of help in the follow-up of individuals as a therapy monitor. The latter is not assessed in this thesis but should be explored in future research. As humming is a new aspect in nNO measurement we think that there is much more to explore. Finally, we can say that there is still a lot to be learned on nNO and that the present knowledge suggests that it has the potential to become a useful tool in diagnosing and/or monitoring upper airway diseases.

Samenvatting

Stikstofoxide (NO) is een gas dat in grote hoeveelheden in de bovenste luchtwegen wordt gevonden. In de lagere luchtwegen zijn de concentraties een honderdvoud lager. Het is niet duidelijk waarom de concentraties NO in de neus en neusbijholten zo veel hoger zijn. Onderzoekers veronderstellen dat de hoge concentratie NO in de neus (nNO) ervoor zorgt dat de bijholten steriel zijn, maar nNO zou ook een rol kunnen spelen in de trilhaar functie van het neusslijmvlies. Daarnaast wordt gesuggereerd dat nNO functioneert als een signaalmolecuul in de luchtwegen ter bevordering van de longfunctie.
De concentratie NO in de longen – c.q. de lage luchtwegen – wordt gebruikt als een aanduiding van eosinofiele ontsteking en ook als monitor van therapie. Zo is bijvoorbeeld de concentratie NO in de longen verhoogd bij iemand met astma en dalen in overeenstemming met de NO waarden tijdens behandeling met inhalatie corticosteroïden. Of nNO ook gebruikt kan worden als een indicator van infectie van bovenste luchtweg aandoeningen, zoals bijvoorbeeld allergische rhinitis, nasale poliepen of meer zeldzaam cystische fibrose (CF, ook wel taaislijmziekte of mucoviscidose genoemd) is nog niet duidelijk. Dat dit nog niet duidelijk is onder andere het gevolg van de verschillende technieken die er zijn om nNO te meten wat vervolgens leidt tot verschillende resultaten. In het algemeen wordt een nNO meting gekenmerkt door een langzame stijging naar een stabiel plateau. Recent is ontdekt dat ‘zoemen’, fonatie van een ‘m’ of een ‘n’, tijdens een nNO meting leidt tot een verandering van de karakteristieke nNO curve zoals gezien tijdens een meting zonder zoemen. In plaats van een langzame stijging is er direct een stijging tot een piekwaarde waarna de nNO concentratie weer geleidelijk afneemt tot een plateau waarde. Wetenschappers suggereren dat de piekwaarde een maat zou kunnen zijn voor de doorgankelijkheid van de neus naar de neusbijholten (het ostiomeataal complex). Derhalve, zou ‘zoemen’ extra (diagnostische) waarde kunnen verstrekken over de doorgankelijkheid van de neus naar de neusbijholten. De werkelijke waarde van de meting tijdens zoemen is niet duidelijk.

In dit proefschrift hebben we onderzocht hoe nNO het best gemeten kan worden en hebben we normaalwaarden voor nNO vastgesteld in kinderen en volwassenen. We hebben het effect van zoemen op nNO in gezonde volwassenen onderzocht om vervolgens te onderzoeken of nNO metingen met en zonder zoemen kunnen bijdragen in de diagnostiek van allergische rhinitis, nasale poliepen en CF.

In **hoofdstuk 1.2** wordt in een overzichtsartikel ingegaan op de oorsprong, productie en meting van NO in de neus. Ook wordt het gevolg van allergische rhinitis, nasale provocatie en medicatie op de concentratie NO in de neus beschreven. We concluderen dat er overeenstemming over de meetmethode van nNO ontbreekt. De expressie van het NO-synthetase, een enzym dat de NO productie induceert, is verhoogt in patiënten met allergische rhinitis en dat het seizoen geen evident effect

heeft op de NO concentraties in de neus. De oorsprong van nNO lijkt vooral de paranasale sinus te zijn, hoewel de discussie omtrent de exacte oorsprong van het gas blijft.

In **hoofdstuk 2.1** wordt het meten van nNO nader onder de loep genomen. Er is gekozen voor een meetmethode die aanbevolen is door de American Thoracic Society, namelijk het meten van nNO tijdens het vasthouden van de adem na een diepe inademing. Er wordt een plastic olijf tegen 1 neusgat geplaatst waarna de proefpersoon verzocht wordt de adem 10 seconden in te houden. Ondertussen wordt actief lucht uit de neus onttrokken welke direct on-line wordt gemeten en weergegeven. De snelheid waarmee de lucht onttrokken wordt (aspiratie snelheid) is van essentieel belang voor de meting omdat de gemeten nNO concentraties daarvan afhankelijk zijn ('flow dependent'); een hogere aspiratie snelheid geeft lagere nNO concentraties. Een goede meting wordt gekenmerkt door een curve van oplopende nNO concentraties tot een plateau. De nNO waarde van een persoon is het gemiddelde van 3 nNO metingen in de laatste 3 seconden (7 – 10 sec) van het plateau. Met deze methode onderzochten we het effect van 3 verschillende aspiratie snelheden (0,28; 0,7 en 1,2 L/min), de neuscyclus, het tijdstip van meting en de reproduceerbaarheid op nNO concentraties. In overeenstemming met de literatuur vonden wij ook dat nNO concentraties afhankelijk zijn van de aspiratie snelheid. Het tijdstip en de neuscyclus hebben geen invloed op nNO concentraties en met een aspiratie snelheid van 0.7 L/min zijn de metingen snel, reproduceerbaar en gemakkelijk uit te voeren.

In **hoofdstuk 2.2 en 2.3** worden de normaalwaarden voor nNO in 340 kinderen en 100 volwassenen vastgesteld. De gemiddelde NO concentratie in de neus van kinderen is 449 ppb (parts per billion). De concentratie wordt bij kinderen jonger dan 12 jaar beïnvloed door de leeftijd en een adenotomie (verwijdering neusamandel). Bij alle kinderen tot 18 jaar wordt nNO beïnvloed door NO in de omgeving (ambient NO). De gemiddelde nNO concentratie in volwassenen (> 18 jaar) is 455 ppb en is niet afhankelijk van NO in de omgeving. Voor alle leeftijden geldt dat de nNO concentraties niet afhankelijk zijn van het geslacht, passief roken en de 'body mass index'.

In **hoofdstuk 2.3** is nNO ook gemeten tijdens zoemen. Uit de groep van 100 volwassenen werden willekeurig 40 deelnemers gevraagd 2 keer achter elkaar te zoemen met een pauze van 1 minuut tussen de metingen. De mediane piekwaarde was 1019 ppb na de eerste keer en 837 ppb na de tweede keer zoemen. Het verschil is significant en is waarschijnlijk het gevolg van een tijdelijk tekort aan NO uit de neusbijholten. De plateauwaarde na de piek tijdens zoemen blijkt gelijk aan de plateau waarde tijdens een 'stille' meting.

Hoofdstuk 3.1. beschrijft de bevindingen van de nNO concentraties met en zonder zoemen in gezonde mensen en in mensen met allergische rhinitis of neuspoliepen. De gemiddelde nNO in patiënten met allergische rhinitis lijkt hoger te zijn dan in gezonde controles maar is net niet significant. De gemiddelde nNO concentratie is significant lager in mensen met neuspoliepen vergeleken met gezonde mensen. De mediaan van de nNO uitkomsten tijdens zoemen waren significant lager in patiënten met neuspoliepen ten opzichte van mensen met allergische rhinitis en gezonde controles. Zoemen lijkt een toegevoegde waarde te hebben ten opzichte van 'stille' nNO metingen, aangezien nNO uitkomsten gemeten tijdens zoemen een beter onderscheid maken tussen gezonden en mensen met neuspoliepen dan 'stille' nNO waarden.

In **hoofdstuk 3.2** kijken we naar nNO waarden met en zonder zoemen in patiënten met CF. CF wordt veroorzaakt door een defect gen wat in de luchtwegen leidt tot vorming van dik, taai slijm. Deels ontstaat dit tengevolge door immobiliteit van de trilharen in de luchtwegen. Het is bekend dat nNO heel laag is in CF patiënten, nNO concentraties tijdens zoemen zijn echter niet eerder gerapporteerd. Wij vonden, in overeenstemming met de literatuur, significant lage 'stille' nNO waarden in CF patiënten ten opzichte van de nNO concentraties in gezonde controles. Ook de nNO uitkomsten tijdens zoemen waren significant lager in CF patiënten. Evenwel lijken ook hier de nNO uitkomsten tijdens zoemen een beter onderscheid te kunnen maken tussen de gezonde controles en CF patiënten dan stille nNO metingen.

In **deel 4** bediscussiëren we de gebruikte methodologie, onze resultaten en doen we suggesties voor toekomstig onderzoek op nNO gebied. Dit proefschrift heeft als doel te onderzoeken of nNO een bijdrage kan leveren in de diagnostiek van bovenste luchtweg aandoeningen. De meting op zichzelf is eenvoudig, snel en makkelijk toepasbaar in de praktijk. Wij zijn van mening dat het nog niet duidelijk is of nNO, met en zonder zoemen, een bijdrage zal kunnen leveren in het diagnostische pad van allergische rhinitis. In tegenstelling tot nNO metingen bij patiënten met neuspoliepen en CF. Hier zou nNO zouden weldegelijk een rol kunnen spelen bij het vaststellen van neuspoliepen en CF. Vooral nNO verkregen tijdens zoemen kan daaraan bijdragen. Hierbij moet wel worden opgemerkt dat de nNO waarden, met en zonder zoemen, van patiënten met neuspoliepen en CF vergelijkbaar (laag) zijn. In de toekomst zou nNO als een screening instrument gebruikt kunnen worden bij deze aandoeningen en in deze hoedanigheid wellicht ook bij allergische rhinitis. Nasaal nNO zou ook een rol kunnen spelen in het volgen (follow-up) van individuen als een therapie monitor. Dit laatste is niet onderzocht in dit proefschrift, maar zou onderzocht moeten worden in verder onderzoek. Aangezien zoemen een nieuw aspect is van nNO metingen is er op dit vlak nog veel te onderzoeken. Uiteindelijk kunnen we zeggen dat er nog veel te leren valt over nNO en dat de huidige kennis suggereert dat nNO de potentie heeft een waardevolle rol te vervullen in de diagnostiek en/of het monitoren van bovenste luchtweg aandoeningen..

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Biography

Veerle Margrethe Diana Struben was born on July 11th 1976 in Rotterdam. In 1994, she graduated from the Rhedens Lyceum" to study medicine at the Erasmus University Rotterdam. During her studies she participated in a research project on Non-alcoholic steatohepatitis and cryptogenic cirrhosis within kindreds (department of Gastroenterology of the University of Virginia, USA) and in an immunohistochemical study on Inhibin subunits and follistatin in pituitary adenomas (department of Immunology, Brookes University, Oxford, GB). She received her Medical Degree in 2001. In the same year she started as a resident / PhD student (AGIKO) at the department of Otorhinolaryngology, Head- and Neck surgery, at the Erasmus Medical Centre in Rotterdam. In 2002, she enrolled in a Master of Science program for Clinical Epidemiology (n i h e s, Rotterdam) and received the MSc degree in 2003. In the same year, she started the project described in this thesis and participated in the PIAMA study (Prevention and Incidence of Asthma and Mite Allergy, multi-centre trial). In December 2005 she voluntarily ended her ENT residency. In January 2006 she started working at NIVEL (Dutch Institute for research in healthcare) were she is involved in a project on patient safety and patient safety culture in hospitals. The author lives together with Onno Roelofs.

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Abbreviations

aNO	Ambient NO
AR	Allergic Rhinitis
ATE	Adenotonsillectomy
BMI	Body Mass Index
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CS	Chronic sinusitis
ECHRS	European Community Respiratory Health Survey
eNO	Exhaled NO
eNOS	Endothelial NOS
ENT	Ear Nose Throat
ESS	Endoscopic Sinus Surgery
HC	Healthy Controls
iNOS	Inducible NOS
ISAAC	International Study of Asthma and Allergies in Childhood
L-NAME	NG-nitro-L-arginine methyl ester
nNO	Nasal NO
nNOS	Neuronal NOS
NO	Nitric Oxide
NOS	Nitric Ocide Synthase
NP	Nasal Polyps
ppb	Parts per billion
RAST	Radio-Allergo-Sorbent-Test
SPT	Skin Prick Test