Fractional exhaled nitric oxide —F_{ENO}— methodology and application in asthma epidemiology in Northern Europe

A dissertation presented
by

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to
The Faculty of Medicine

in partial fulfilment of the requirements
for the degree of
Doctor of Philosophy

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University of Helsinki
Helsinki, Finland
October 2021

Doctoral thesis, to be presented for public examination
with the permission of the Faculty of Medicine
of the University of Helsinki in Suomen Laki Hall,
Porthania, Yliopistonkatu 3, 00170 Helsinki,
on the 30th of October, 2021 at 12 o'clock c.t.

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Cover art by Paul G. Lassmann-Klee.
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Cover layout by Anita Tienhaara.
Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis No.64/2021
ISSN 2342-3161 (print)
ISSN 2342-317X (online)
ISBN 978-951-51-7633-2 (paperback)
ISBN 978-951-51-7634-9 (PDF)
https://ethesis.helsinki.fi
Printed in Helsinki by Unigrafia, 2021

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Abstract

In Northern Europe, the prevalence of asthma differs between countries, but an objective clinical comparison of bronchial inflammation is missing. Therefore, we aimed to study the fractional exhaled nitric oxide F_{ENO}, a biomarker of airway inflammation, in population samples of the general populations of Sweden, Finland and Estonia. We further aimed to analyse methodological and physiological features of F_{ENO} acquisition, such as mouthwashes and dependency of expiratory flow.

We performed clinical interviews (n = 2658), F_{ENO} (n = 1498) and skin prick tests (SPT) in a random population from Sweden (Stockholm and Örebro), Finland (Helsinki), and Estonia (Narva and Saaremaa), during 1997–2003. To analyse the methodology of F_{ENO}, we performed two pilot clinical studies. Firstly, we measured F_{ENO} with an expiratory flow rate of 50 mL/s in a small random sample (12 asthmatic or healthy) and acquired a baseline, then repeated measurements (for 20 min) after a mouthwash with tap water or carbonated water. Additionally, we obtained F_{ENO} from 30 volunteers for multiple expiratory flow rates of 50, 30, 100 and 300 mL/s, after different mouthwash settings. With this dataset, we analysed the influence of mouthwashes in multiple-flow F_{ENO} and developed a conversion model, with a further cross-validation in five populations: healthy adults, healthy children, and patients with chronic obstructive pulmonary disease (COPD), asthma and alveolitis.

In the pilot studies, the tap water mouthwash reduced F_{ENO} for only 2 min. The mouthwash with carbonated water lowered F_{ENO} more notably, for 12 min. Compared to the tap water mouthwash, the carbonated water mouthwash reduced F_{ENO} at all expiratory flows — 50, 30, 100 and 300 mL/s. The carbonated water mouthwash also lowered the maximum airway NO flux ($\dot{J}_{awNO}$), but not the alveolar NO concentration (C_{ANO}) or capacity of the airways for NO diffusion (D_{awNO}). We developed a non-linear model to perform F_{ENO} estimations obtained at different flows. The cross-validation resulted in a low deviation between estimated $\hat{F}_{ENO}$ (from 100 mL/s to 50 mL/s) and measured F_{ENO} (at 50 mL/s) in children (0.27 ppb), the mixed adult population (0.28 ppb), and in healthy adults (0.44 ppb). The deviation was higher in patients with COPD (1.16 ppb), alveolitis (1.47 ppb), and asthma (1.68 ppb). We applied the non-linear model to standardise the F_{ENO} values at 50 mL/s in the epidemiological study.

In the population study, the median (interquartile range) of F_{ENO} (ppb) was 15.5 (9.3) in Sweden, in Finland 15.4 (13.6), and in Estonia 12.5 (9.6). We found the lowest F_{ENO} in Estonian centres —Saaremaa 13.1 (9.5) and Narva 11.8 (8.6). Asthma was associated with $F_{ENO} \geq 25$ ppb, odds ratio (OR) 3.91 (95% confidence interval: 2.29–6.32) and adjusted for skin prick test (SPT),

smoking, sex and study centre. Atopy increased the likelihood of asthma, OR 3.19 (2.02–5.11). Having asthma was more likely in Stockholm OR 5.54 (2.18–14.79), Örebro OR 3.38 (1.59–8.09), Helsinki OR 2.40 (1.04–6.02), and Narva OR 2.45 (1.05–6.19), compared to Saaremaa.

We conclude that a carbonated water mouthwash reduces oral NO contamination for 12 min, and is more pronounced than tap water, with multiple flows, without affecting C_{ANO} or D_{awNO}. We established a model for converting F_{ENO} between multiple expiratory flows, with further tentative use of predicting extended flow parameters, $\dot{J}_{\mathrm{awNO}}$ and C_{ANO}. We confirmed the higher prevalence of allergic airway inflammation and asthma in Sweden and Finland, compared to Estonia. An increased F_{ENO} and atopy were independently associated with a higher risk of asthma. Our epidemiological findings support the west–east disparity of allergic diseases.

List of original publications, referred to in the text by their Roman numeral:

I Lassmann-Klee PG, Lindholm T, Metsälä M, Halonen L, Sovijärvi ARA, Piirilä P. Reduction of F_{ENO} by tap water and carbonated water mouthwashes: magnitude and time course. *Scand. J. Clin. Lab. Invest.* 2018; 78: 153–156.
https://doi.org/10.1080/00365513.2017.1419574

II Lassmann-Klee PG, Lehtimäki L, Lindholm T, Malmberg LP, Sovijärvi ARA, Piirilä P. Influence of mouthwashes on extended exhaled nitric oxide (F_{ENO}) analysis. *Scand. J. Clin. Lab. Invest.* 2018; 78: 450–455.
https://doi.org/10.1080/00365513.2018.1497802

III Lassmann-Klee PG, Lehtimäki L, Lindholm T, Malmberg LP, Sovijärvi ARA, Piirilä PL. Converting F_{ENO} by different flows to standard flow F_{ENO}. *Clin. Physiol. Funct. Imaging* 2019; 39: 315–321.
https://doi.org/10.1111/cpf.12574

IV Lassmann-Klee PG, Piirilä PL, Brumpton B, Larsson M, Sundblad B-M, Põlluste J, Juusela M, Rouhos A, Meren M, Lindqvist A, Kankaanranta H, Backman H, Langhammer A, Rönmark E, Lundbäck B, Sovijärvi ARA. Parallel gradients in F_{ENO} and in the prevalences of asthma and atopy in adult general populations of Sweden, Finland and Estonia —a Nordic EpiLung study. *Respiratory Medicine* 2020; 173, 106160.
https://doi.org/10.1016/j.rmed.2020.106160

Abbreviation list

AIC	Akaike information criterion
ATS	American Thoracic Society
BHR	Bronchial hyperreactivity
$CaCl_2$	Calcium chloride
CI	95% Confidence interval
COPD	Chronic obstructive pulmonary disease
C_v	Confidence of variation
ECP	Eosinophilic cationic protein
F_{ENO}	Fractional exhaled nitric oxide
GLM	General linear model
ICS	Inhaled corticosteroids
IL	Interleukin
IQR	Interquartile range
LABA	Long-acting β_2-agonist
MD	Medical doctor
$NaHCO_3$	Sodium hydrogen carbonate
NO_2	Nitrogen dioxide
NO_3^-	Nitrate
Th_2	T helper 2 CD_4^+ cells
O_2-	Superoxide anions
OR	Odds ratio
ppb	Parts per billion
RNS	Reactive nitrogen species
SABA	Short-acting β_2-agonist
SE	Standard error
UK	United Kingdom
ARC	Allergic rhinitis or conjunctivitis
BAL	Bronchoalveolar lavage
BMI	Body mass index
C_{ANO}	Alveolar nitric oxide concentration
cNOS	Constitutive nitric oxide synthase
CRP	C reactive protein
D_{awNO}	Airway nitric oxide diffusion
ERS	European Respiratory Society
FEV_1	Forced expiratory volume in 1 s
HMA	Högman and Meriläinen algorithm
IgE	Immunoglobulin E
iNOS	Inducible nitric oxide synthase
$\dot{J}_{awNO}$	Maximum airway nitric oxide flux
MAD	Median absolute deviation
$MgCl_2$	Magnesium Chloride
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase
$\dot{V}$	Flow rate
ONOO-	Peroxynitrite
pH	Potential of hydrogen
RCT	Randomised controlled trail
ROS	Reactive oxygen species
SD	Standard deviation
SPT	Skin prick test

Contents

Listing of figures

To my family

Ὁ βίος βραχύς, ἡ δὲ τέχνη μακρή, ὁ δὲ καιρὸς ὀξύς, ἡ δὲ πεῖρα σφαλερή, ἡ δὲ κρίσις χαλεπή

Vīta brevis, ars longa, occāsiō praeceps, experīmentum perīculōsum, iūdicium difficile.

Life is short, and art long, opportunity fleeting, experimentations perilous, and judgment difficult.

Hippocrates of Kos

0

Introduction

Asthma, from ancient Greek ἆσθμα (āsthma), probably derives from an Indo-Germanic root (*ane-) meaning breath, and is characterised by breathing difficulties (Kluge, 2019). The oldest records describing asthma-like conditions originate from China (Unschuld et al., 2011) and Egypt (Ebers, 1875). Although we ignore whether these historical descriptions of asthmatic conditions resemble our modern disease entity, this certainly suggests that asthma-like conditions have afflicted humanity at least since the beginnings of history. Modern definitions of asthma include the nature of its acute episodic symptomatic, either with concomitant in-

flammation, or with structural changes, or both, eventually causing persistent symptoms and impaired respiratory function (Reddel et al., 2009). Current medical approaches, like diagnosis and therapy, are based on the aforementioned concept of asthma disease. The dichotomy of cause and cure prevails in medical practice, and clinicians routinely search for evident (phenomena) signs of the disease, establish the probable cause and implement the treatment. In the case of asthma, current research has slowly disentangled a multi-factorial cause and proposed treatment approaches. We are centuries beyond treating asthma with tobacco cigars, and decades from only prescribing β_2-agonists. These inhaled β_2-agonistic drugs, refined and combined with corticosteroids, became a milestone in asthma treatment as soon as the inflammatory component of the disease was recognised (Haahtela et al., 1991). Advances in medical research have provided us with numerous treatable traits and an arsenal of drugs (e.g., monoclonal antibodies). As suggested by Anderson (2008), asthma is just a label for many different pathological entities (endotypes) caused by different pathophysiological processes exhibiting differences in diagnostics and responding to divergent regimes. Therefore, rethinking what asthma is and deconstructing a unique asthma label was previously proposed (Pavord et al., 2018). Although the discovery of many potential diagnostic biomarkers of different asthma phenotypes is ongoing, adopting these biomarkers in clinical practice is a slow, but continuous process. In this context, almost 30 years have passed since the discovery of nitric oxide (NO) production in the lungs by Gustafsson et al. (1991) and the development of fractional exhaled nitric oxide (F_{ENO}) into a clinical tool, not officially included in national asthma guidelines until recently (National Institute for Health and Care Excellence, 2017). The methodology of F_{ENO} measurement has constantly improved, although research has shown its limitations. Thus, finding an optimal method of diagnosing asthma resembles searching for the holy grail and implies a Sisyphean task. Most likely, an array of established methods will emerge to classify and diagnose different asthma endotypes, and become accessible in most clinical settings. The reader might ask why diagnosing asthma easily and effectively is imperative? Asthma is a common disease with 3.4 million cases worldwide (Vos et al., 2017) among all ages. Therefore,

its treatment is associated with large health expenses, and despite therapeutic advances, asthma causes years of disability and deaths. Furthermore, developing adequate diagnostic procedures tailored to identify patients at high risk of asthma and defining which endotype causes their disease could ameliorate the societal costs through individualised therapies. Similarly, it is of high interest to analyse which populations have a higher asthma risk and which factors can cause asthma during the life-course. The study of diseases in populations — epidemiology — facilitates comparisons between groups, risk factors estimates; as when using para-clinical tools like laboratory tests, epidemiology can find associations between abnormal values and a higher risk for diseases. During the 1990s, epidemiological research found, when comparing the rates of allergic diseases between West and East Germany, a disparity with a higher prevalence of asthma in West Germany (von Mutius et al., 1994). These findings led to postulating the 'Hygiene hypothesis', which attributes the high prevalence of allergic diseases to hygienic living conditions in high income countries wherein people often lack the exposure to certain environmental factors. Currently, a multifactorial aetiological hypothesis provides a more accurate framework to explain the protective or risk factors of asthma. But how do F_{ENO} and the west–east disparity of asthma relate? Utilising F_{ENO} to assess airway inflammation and comparing the results between countries could verify or refute the west–east * disparity of asthma without relying only on questionnaires in epidemiological research. Although many questions will remain unanswered, reading this thesis will provide a deeper insight into the methodology of F_{ENO} measurement with new aspects not previously investigated, and on how applying F_{ENO} to populations from Northern European countries offers new insight into asthma epidemiology.

Paul G. Lassmann-Klee

* The west–east disparity does not refer to a strict geographical classification, but to historical differences in Europe, with 'eastern'countries belonging to a former socialist Eastern Bloc under the Soviet Union and 'western'countries classified as a capitalist Western Bloc.

A journey of a thousand miles begins with a single step

Lao-Tze

1

Literature overview

1.1 Physiology and pathophysiology of nitric oxide

In 1991, Gustafsson et al. (1991) observed nitric oxide (NO) in exhaled air, known as fractional exhaled nitric oxide (F_{ENO}), experimentally in humans and animals. During the following years, Alving et al. (1993) reported increased F_{ENO} values in asthmatics, and further researchers studied the origin of respiratory NO by applying NO synthase inhibitors, as well as the reducing effect of corticosteroids on F_{ENO} in asthmatics (Kharitonov et al., 1994; Massaro et al., 1995;

Yates et al., 1995). These early investigations suggested a main synthesis of F_{ENO} through an inducible NO synthase (See panel 1) after the discovery of two isoforms of NO synthase enzymes, an inducible (iNOS) and a constitutive NO synthase (cNOS) (Radomski et al., 1990).

Palmer et al. (1987) first demonstrated the vasodilation effect of NO produced by endothelial cells and its synthesis from L-arginine (Palmer et al., 1988). NO similarly acts as a vasodilator during hypoxia in the lungs (Liu et al., 1991) and can up-regulate ciliary motility (Jain et al., 1993), therefore playing an important role in lung physiology. However, bronchodilation — as well as other physiological effects like anti-inflammation — is mediated by the cNOS, as opposed to the effects of NO synthesised by iNOS, whose role in inflammatory processes leads to activation of beneficial host defence mechanisms, but is also involved in pathophysiological processes observed in asthma: infiltration of immune cells, activation of T helper 2 cells (Th2), augmentation of vascular permeability, over-production of mucus, remodelling of the airway tissue, and contraction of smooth myocytes, among others (Ricciardolo, 2003).

In asthmatic lungs, the bronchial tissue and inflammatory cells overexpress iNOS and produce reactive stress oxidants — superoxide anions (O_2^-), peroxynitrite ($ONOO^-$) — with detrimental effects (Saleh et al., 1998). On the other hand, to some physiological extent, activation of iNOS can have a beneficial effect (Schuiling et al., 1998), and some researchers have proposed a NO homeostasis, i.e., a balance between cNOS and iNOS (Meurs et al., 2003). A disruption of this homeostasis, through deficient cNOS and production of oxidants from iNOS, is involved in the pathophysiology of asthma. This disequilibrium of the NO synthases' activation may partly be caused by low levels of the substrate L-arginine, leading to reactive oxygen species (ROS) and reactive nitrogen species (RNS) production (Wells and Holian, 2007) and the involvement of asymmetric dimethylarginine, an inhibitor of NOS. Both iNOS and cNOS, and also other isoforms of NO synthase are expressed in the airway epithelial cells and in the alveolar type II cells (Asano et al., 1994), therefore contributing to the total F_{ENO} in the lungs. It seems plausible that the NO originates in the whole respiratory tract, however, the production in the lower airways accounts for the increased F_{ENO} found in asthmatics (Kharitonov et al.,

1996; Massaro et al., 1996). Within the respiratory tract, sources of higher amounts of NO are the nasal (Lundberg et al., 1995; Törnberg et al., 2002) and oral cavities (Marteus et al., 2005; Olin et al., 2001). Therefore, F_{ENO} acquisition methodology includes techniques to avoid contamination (oral and nasal) while applying them in asthma diagnostics, as explained later.

Panel 1. Inducible nitric oxide synthase (iNOS)

Inducible NO synthases (iNOS), also known as nitric oxide synthase 2, coded in human chromosome 17 can be expressed by virtually any cell (NCBI, 2021), but are found mainly in macrophages as part of the physiological immune response against parasites, viruses, bacteria and tumour cells. Certain cytokines and lipopolysaccharides can induce macrophages to synthesise NO, but other cells, e.g., endothelial cells, can also have cytotoxic effects through action of NO. NO has an affinity for protein-bound iron and therefore can interfere with the electron transport and metabolism of mitochondria, with DNA replication, and in higher concentrations can cause DNA fragmentation. The other side of the coin of this immune defence is cytotoxicity of neighbouring cells, and tissue damage through NO or ONOO-(Förstermann and Sessa, 2012).

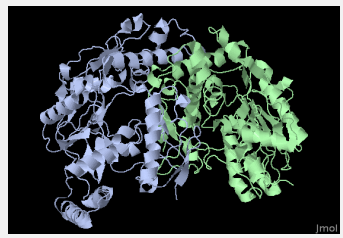


Figure 1.1: Structure of inducible nitric oxide synthase. Data from Fischmann et al. (1999), structure visualised with MATLAB ®software, MathWorks ®

The oral microflora can reduce dietary or salivary nitrate (NO_3^-) to nitrite (NO_2^-) (Duncan et al., 1995). These facultative anaerobic bacteria possess a nitrate reductase that catalyses this enzymatic reaction (Eq. 1.1). NO_2^- can be further denitrified to NO by biological NO_2^- reduction (Eq. 1.2) or chemical acidic decomposition of NO_2^- (Eq. 1.3), the latter being pH-dependent (pH<5) (Hezel and Weitzberg, 2015; Schreiber et al., 2010).

$$NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O \tag{1.1}$$

$$NO_2^- + 2H^+ + 2e^- \longrightarrow NO + H_2O \tag{1.2}$$

$$\begin{aligned} NO_2^- + H^+ &\longrightarrow HNO_2 \\ 2HNO_2 &\longrightarrow N_2O_3 + H_2O \\ N_2O_3 &\longrightarrow NO + NO_2^- \end{aligned} \tag{1.3}$$

Apart from the NOS dependent pathways, the human body can form NO in a NOS-independent manner, a reduction of NO_2^- through several enzymes (Lundberg et al., 2008). This physiological pathway may have beneficial effects on several organs, where the above-mentioned microflora provides nitrite in a symbiotic relationship with the human host.

A recent study found different genetic expression patterns in the epithelium between upper and lower airways, supporting the view of different local patterns of chronic airway inflammation, due to local production of Th2 cytokines (Vieira Braga et al., 2019). In allergic asthmatics, the lower airway mucosa is infiltrated by eosinophils, Th2 and mast cells (Brown et al., 2007). In asthma, Th2 cells secrete interleukins (IL), such as IL-4, IL-5 (Lee et al., 1997), IL-9, IL-13, IL-25 and IL-31 (Nakajima and Takatsu, 2007) or interleukins can activate Th2 cells, e.g., IL-4 (Coyle et al., 1995). Targeting some of these interleukins with monoclonal antibodies have shown a F_{ENO} reduction, implying an important role of Th2 cells in the pathophysiology of F_{ENO}. Similarly, immunoglobulin E (IgE), produced by secretory B-cells and activating eosinophils, mast cells, and basophils, is ligated to F_{ENO}. Activated eosinophils damage the bronchial epithelium by releasing certain proteins — major basic protein and eosinophilic cationic protein (Venge et al., 1988). Anti-IgE drugs reduce F_{ENO} and represent a therapeutic pathway in allergic asthma (Hanania et al., 2013). Other biological drugs show that other cells, such as dendritic cells and mast cells, are involved in F_{ENO} pathophysiology (Corren et al., 2017). The role of therapeutics and the different asthma phenotypes will be discussed in the following subsections.

As stated before, the lower airways are mainly responsible for the increased F_{ENO} in asthma, but the flow dynamics within the airways also determine the final NO concentration in the exhaled air. Within this context, the expiratory flow rate is a crucial factor affecting the level and source of F_{ENO} in the final exhaled air. Other factors influencing F_{ENO} are the exhalation time and exhaled air volume. Mathematical models are useful in predicting F_{ENO} at any exhalation rate, employing flow-independent NO parameters. For example, a two-compartment model consisting of alveolar and airway spaces employs the alveolar NO concentration (C_{ANO}), the capacity of the airways of NO diffusion (D_{awNO}) and the maximum airway NO flux ($\dot{J}_{\text{awNO}}$) (George et al., 2004) for modelling F_{ENO}. The two-compartment model is based on the first law of diffusion by Fick (1858)*. These models emulate the physiology of F_{ENO} within the lungs and consider the main factors contributing to F_{ENO} dynamics. The extended parameters reveal an increased $\dot{J}_{\text{awNO}}$ in asthmatics, i.e., a higher airway NO flux (Högman et al., 1999; Lehtimäki et al., 2000). Further, $\dot{J}_{\text{awNO}}$ relates to bronchial hyperreactivity and markers of serum eosinophilia (Lehtimäki et al., 2001).

1.2 Methodology of F_{ENO} measurement

1.2.1 F_{ENO} analysers

Nitric oxide is a short-lived molecule due to its high reactivity and low concentration — picomolar to nanomolar — in human breathing (Ricciardolo et al., 2015). In order to detect NO, analysers utilise a chemiluminescent reaction (Eqs. 1.5 and 1.6) to quantify NO (Stedman et al.,

*In this case the flux is the net movement of molecules from a high concentration to a low concentration region, i.e., the flux J is proportional to the concentration gradient and dependent on the diffusion coefficient D, formulated as

$$J = -D\frac{\partial c}{\partial x} \tag{1.4}$$

1972), which was originally applied to detect ozone or nitric oxide in the atmosphere.

$$O_3 + NO \longrightarrow NO_2^* + O_2 \tag{1.5}$$

$$NO_2^* \longrightarrow NO_2 + h\nu \tag{1.6}$$

Within the chemiluminescent reaction, NO reacts with ozone from an ozone generator and forms nitrogen dioxide (NO_2^*), but as marked by the asterisk in the notation, nitrogen dioxide has an additional electron and is therefore in an unstable and excited state. By spontaneously returning to a relaxed state, it releases a photon ($h\nu$). This light can be quantified by a photomultiplier and the intensity is related to the concentration of NO in the probe. Chemiluminescent analysers are very sensitive and have a fast response, but are costly and large (Maniscalco et al., 2016). Another analyser type of more recent development is based on an electrochemical reaction. These analysers are handheld, portable F_{ENO} measuring devices with lower acquisition costs. The first F_{ENO} handheld device was developed in 2004 by Hemmingsson et al. (2004). These analysers utilise amperometric gas sensors, which are electrochemical cells, with a membrane, two electrodes and an electrolyte (Cao et al., 1992). The main reaction is based on the electron transfer from nitric oxide from the breath probe to the electrode, see 1.7. This creates a measurable electric current proportional to the concentration of NO.

$$NO + 2H_2O \longrightarrow NO_3 + 4H^+ + 3e^- \tag{1.7}$$

The commercially available F_{ENO} electrochemical analysers have the disadvantage of frequent sensor cell replacement Maniscalco et al. (2016).

1.2.2 Expiratory flow

As mentioned before, F_{ENO} is highly dependent on the expiratory flow. At high expiratory flow, F_{ENO} values are low. Lowering the flow rate causes an increase in F_{ENO} values. Expira-

Table 1.1: Non-exhaustive list of models in extended F_{ENO} analysis

Model	Number of flow rates	Flow rate range (mL/s)	Reference
Silkoff technique	9	4–1550	(Silkoff et al., 2000)
Tsoukias and George	6	100–500	(Tsoukias and George, 1998)
Pietropaoli technique	6	6–1355	(Pietropaoli et al., 1999)
Högman and Meriläinen algorithm	3	10–500	(Högman et al., 2000)
Trumpet model axial diffusion	4	100–250	(Condorelli et al., 2007)
Eckel nonlinear logarithmic	4	30–300	(Eckel et al., 2014)

tory flow rate and F_{ENO} are inversely proportional, and a plausible explanation attributes this dependency to the passage time of the expiratory air at the bronchial wall, from which NO is diffused (Silkoff et al., 1997). For this reason, to ensure comparability, the American Thoracic Society (1999) (ATS) adopted a standard flow value of 50 mL/s. This value aims to provide F_{ENO} from the bronchi with a comfortable exhalation technique, since expiration at higher flow rates can be difficult for some individuals. Additionally, much higher flow rates (>200 mL/s) acquire F_{ENO} mainly from the alveolar space.

Previous recommendations prior to standardisation and the now widely accepted 50 mL/s flow rate included flow rates between 83 and 250 mL/s (Kharitonov et al., 1997). A constant flow at a desired flow rate is usually achieved through the use of a fixed resistance, against which the test person exhales. The relationship between resistance, expiratory flow rate and expiratory pressure behaves like Ohm's law (for Newtonian fluids with laminar flow). If setting a fixed resistance, the expiratory flow rate is directly proportional to the expiratory pressure applied, and modern F_{ENO} measuring devices include a visual feedback so the test person can apply a constant expiratory pressure and a constant flow rate effectively. The positive expiratory pressure (of 5–20 cm H_2O) ensures velum palatinum closure, with the essential purpose to avoid NO contamination from the nose into the airways, which would affect the results (Silkoff et al., 1997). The standardised flow of 50 mL/s does not apply to the extended NO analysis, since multiple flow rates are usually needed to provide values for the flow-independent parameters: C_{ANO}, D_{awNO} and $\dot{J}_{awNO}$ (Tsoukias and George, 1998). The number of exhalations and different flow rates depend on the specific model employed (See Table 1.1).

1.2.3 Mouthwashes

Oral bacteria may produce NO from dietary nitrate and interfere with F_{ENO}. Although previous reports indicate the use of mouthwashes to reduce oral NO production, current guidelines recommend mouthwashes only in physiological research and not in clinical settings (Horváth et al., 2017), implying minimal interference with the results. The previous section on the physiology of F_{ENO} elucidated the role of oral bacteria in NO production, and investigations showed the effect of chlorhexidine, an antibacterial mouthwash, on F_{ENO} (Heijkenskjöld-Rentzhog et al., 2012; Zetterquist et al., 1999).

Other mouthwashes have been tested with either alkaline solutions and a short effect on F_{ENO} reduction (Zetterquist et al., 1999), or neutral pH (phosphate buffer saline, distilled water) with no effect on F_{ENO}. On the other hand, highly acidic mouthwashes show an increase in F_{ENO} (Zetterquist et al., 1999). Piirilä et al. (2012) earlier showed promising results of a mouthwash with mineral carbonated water with a mildly acidic pH, and the Laboratory of Clinical Physiology in the Helsinki University Hospital has endorsed its use in routine clinical F_{ENO} testing since 1997.

1.2.4 Repeatability of F_{ENO}

Repeated F_{ENO} measurements may exhibit a variability of obtained values. According to a study by Ekroos et al. (2002), the 10 min reproducibility of F_{ENO} measurements is good in healthy people and asthmatics, but the short-term variability (up to 24 h after baseline) in asthmatics is twice as high as in healthy individuals.

1.2.5 F_{ENO} guidelines

Current F_{ENO} technical guidelines include a joint statement by the American Thoracic Society and European Respiratory Society (2005) with an update on some issues (Horváth et al., 2017). The first F_{ENO} guidelines first appeared in 1997, as an European Respiratory Society (ERS)

task force report (Kharitonov et al., 1997), followed by a recommendation from the ATS from 1999, the latter already endorsing a flow rate of 50 mL/s (Silkoff, 1999). When acquiring F_{ENO}, certain technical standards should be observed. NO-free air should be used for inspiration, since ambient air can contain high levels of NO †. The use of a nose-clip and breath-holding is discouraged, since both increase F_{ENO} through nasal contamination (Kimberly et al., 1996; Sato et al., 1996).

Panel II. Technical highlights of F_{ENO} measurement

- Exhalation with standard flow rate, 50 mL/s (Extended NO analysis employs other flow rates).
- Nitric oxide concentration from plateau's mean value (plateau for 3 s).
- 2 single determinations (minimum) for an acceptable measurement, either 2 plateau values without a 10% difference or more (chemiluminescence analyser) or 2 measurements without a 10% variation (electrochemical devices).
- No spirometry before F_{ENO}.
- No nitrate-containing meals before measurement (2 h).
- No eating, drinking, exercising or smoking (1 h).

Figure 1.2: ECOMEDICS ®CLD 88sp F_{ENO} chemiluminescence analyser (with permission from ECOMEDICS AG, Switzerland).

F_{ENO} values are also influenced by other factors, such as age, sex, viral infections, circadian patterns and drugs. These are to be considered when acquiring F_{ENO}. The most salient technical features are summarised in Panel II above.

†Prof. Sovijärvi measured extreme ambient NO (1–600 ppb) during the winter in Helsinki, Finland (Kharitonov et al., 1997)

1.2.6 F_{ENO} REFERENCE VALUES

According to the F_{ENO} ATS guidelines (Dweik et al., 2011), F_{ENO} results should be interpreted with cut-points in adults. A low F_{ENO} of <25 ppb usually indicates a low probability of eosinophilic inflammation and response to corticosteroids. Values > 50 ppb suggest eosinophilic inflammation, and symptomatic patients could respond to a therapy with corticosteroids. On the other hand, intermediate values (between ≥25 and ≤50 ppb) are to be interpreted in a clinical context. However, apart from applying rigid cut-off values, publications indicate certain characteristics that may influence F_{ENO} levels in healthy and asthmatic individuals.

A systematic review by Jacinto et al. (2013) accounted for the main factors influencing F_{ENO} as age, height, weight, gender, atopy, smoking, and ethnicity. Olin et al. (2007) predicted F_{ENO} reference values based in age and height, but adjusting only for age and height explained only 11% of the variation. In healthy non-smokers, Torén et al. (2017) found that gender, age, height and atopy are decisive factors of F_{ENO} levels and presented reference equations for healthy adults. Similarly, Malmberg et al. (2006) introduced reference values for children, adjusted with height as a main determinant of F_{ENO}. The Reveno study found F_{ENO} differences between men and women in healthy non-smokers (Olivieri et al., 2006). According to large population studies, F_{ENO} in healthy non-smokers increases with age and height (Brody et al., 2013), and shows an increase during childhood due to somatic growth, with a plateau in adulthood and further minor rise at middle age (Jacinto et al., 2015). These general population studies indicate a threshold for a high risk of inflammation, expressed as the upper 95% percentile of the adult population, with a F_{ENO} value of 39 ppb (See and Christiani, 2013).

As mentioned before, smoking and atopy are well-known factors affecting F_{ENO} levels (Taylor et al., 2007). Cigarette smoking reduces F_{ENO} presumably through inhibition of NO synthase, and current smokers have lower F_{ENO} compared to controls (Kharitonov et al., 1995). A systematic review by Ahovuo-Saloranta et al. (2019) found a higher F_{ENO} in untreated asthmatics who were current smokers, compared to healthy individuals who smoke, indicating that

F_{ENO} is applicable regardless of smoking status. F_{ENO} can also differentiate between asthmatics and patients with asthma-like symptoms in never smokers, and also in current smokers (Malinovschi et al., 2012), but cut-off values may need adjustment in smokers. Another study by Rouhos et al. (2010) stratified asthmatics by atopic status and reported that in symptomatic asthmatics, smoking lowers F_{ENO} only in atopic individuals, and not in non-atopic. The same study reported a F_{ENO} median of 29 ppb in non-smoking atopic asthmatics (steroid-naïve) (Rouhos et al., 2010). It should be noted that the multiple variables affecting F_{ENO} in adults are not part of clinical practice and cut-points are used in interpreting F_{ENO} results regardless of atopy, smoking status, gender, height and age.

Högman et al. (2009) published extended F_{ENO} reference values for a healthy random population. This study suggested physiological ranges for F_{ENO} measured at 50 mL/s of 10–30 ppb, for alveolar NO concentrations (C_{ANO}) of 0–4 ppb, for the capacity of the airways of NO diffusion (D_{awNO}) of 5–15 mL/s and for the maximum airway NO flux ($\dot{J}_{awNO}$) of 0.8–1.6 nL/s.

1.2.7 F_{ENO} IN ASTHMA

Acquiring F_{ENO} while performing a clinical work-flow in asthma may provide additional diagnostic value apart from more established methods, like spirometry, peak expiratory flow, bronchial hyperresponsivity (BHR), bronchial biopsies, and induced sputum analysis. Current recommendations from the ATS advocate F_{ENO} use as auxiliary when objective evidence in asthma diagnosis is needed (Dweik et al., 2011). Recent national UK guidelines introduced F_{ENO} as an objective diagnostic tool in routine asthma assessment of symptomatic adults ($\geq$ 17 years), after clinical history and physical examination, and F_{ENO} followed by a spirometry (National Institute for Health and Care Excellence, 2017). On the other hand, the Global Initiative for Asthma (2020) states that F_{ENO} cannot confirm or reject an asthma diagnosis, since it may also be elevated in other conditions — allergic rhinitis, eosinophilic bronchitis, atopy — but might provide additional information regarding treatment in certain patients.

A meta-analysis by Karrasch et al. (2017) from 26 studies found an overall sensitivity of 0.65 (95% CI 0.58 –0.72) and specificity of 0.82 (95% CI 0.76 –0.86), indicating the sufficient diagnostic power of F_{ENO} to confirm asthma and poor ability to reject it [‡]. Wang et al. (2018) also performed a meta-analysis — from 43 studies and 13 747 patients — and found a moderate diagnostic performance of F_{ENO} in ≥5-year-old individuals. The accuracy depended on the cut-off F_{ENO} value used. For example, in adults a cut-off between 30 ppb and 39 ppb had a sensitivity of 0.53 and a specificity of 0.85.

Reported findings in a prospective study suggest that very high F_{ENO} values could predict future asthma risk and exacerbations (Zeiger et al., 2011). Although expert commissions and guidelines argue for or against the use of F_{ENO} as a diagnostic examination, an undoubted merit of F_{ENO} testing lies in assessing certain types of airway inflammation, which can be present in asthma, as discussed below.

Assessment of airway inflammation

The ATS suggests the use of F_{ENO} in diagnosing eosinophilic airway inflammation and monitoring airway inflammation in asthmatics (Dweik et al., 2011). The ATS guidelines regard F_{ENO} changes of 20% as a significant increase or reduction if F_{ENO} are over 50 ppb, or a significant change of 10 ppb if the F_{ENO} values are lower than 50 ppb. In severe asthma, Global Initiative for Asthma (2020) describes F_{ENO} as a possible tool to identify a Type 2 inflammation and for consideration of biological treatment. Type 2 inflammation, as described above (Sec.1.1), involves activation of Th2 cells and exhibits eosinophilia (Fahy, 2015). A previous study found a

[‡]In this specific case, the rate of false negatives is 35% and the rate of false positives 18%. With a two-by-two table, sensitivity (Eq. 1.8) and specificity (Eq. 1.9) of a diagnostic test can be compared to a known condition or another test (usually a gold standard) in a population.

$$Sensitivity = \frac{true\ positives}{true\ positives + false\ positives} \tag{1.8}$$

$$Specificity = \frac{true\ negatives}{true\ negatives + false\ negatives} \tag{1.9}$$

positive association between sputum eosinophilia and F_{ENO}, even at very low F_{ENO} values (> 8.3 ppb) (Berry et al., 2005).

A rise in F_{ENO} may indicate an activation of the inflammatory process in the airways after allergen exposure, with immediate or late bronchoconstriction (assessed by a BHR test), as showed in patients with suspected occupational asthma (Piipari et al., 2002). Within this selected population, negative BHR challenge tests were not associated with changes in F_{ENO}. Interestingly, patients with a high baseline F_{ENO} showed no changes after allergen exposure.

Contribution to asthma treatment

Various studies incorporated F_{ENO} in asthma treatment algorithms and investigated two important aspects, among others. Firstly, the ability of F_{ENO} to guide the treatment, and secondly, the clinical improvement of F_{ENO} guidance (Gibson, 2009). Treatment guidance means the decision to implement a certain treatment, adjust the medication, i.e., the drug type and dose, and initiate or stop a treatment. Asthma algorithms usually contain objective lung-function and allergy tests, apart from classification of symptoms — duration, frequency, type — and frequency of bronchodilator drugs as needed (short-acting β_2-agonists, SABA). The corner stone of modern pharmacological therapy for asthma is inhalation of a combination of drugs, long-acting β_2-agonists (LABA) and corticosteroids (ICS), employed in episodic or continuous use (Global Initiative for Asthma, 2020). Smith et al. (2005a) reported that patients with undiagnosed respiratory symptoms and high F_{ENO} values responded to ICS, and showed a reduction of ICS doses based on F_{ENO} values with equivalent outcomes, compared to conventional guidelines in a single-blind, placebo-controlled trial (Smith et al., 2005b). Another study, in a primary care setting, demonstrated a decrease in ICS use in a F_{ENO}- and symptom-guided regime, without influencing the severe asthma exacerbation rate (Honkoop et al., 2015). Nevertheless, in F_{ENO}-guided therapy regimes, asthma exacerbations were reduced in pregnant women, with improved quality of life and reduction of neonatal hospitalisation (Powell et al., 2011). Accord-

ing to a meta-analysis, F_{ENO}-guided therapy can reduce asthma exacerbations in adults and children (Petsky et al., 2018). A longitudinal study found a relationship between F_{ENO} and asthma control over time. A randomised controlled trial with a one-year follow-up in children found no higher corticosteroid use in a F_{ENO}-controlled regime, but improvement in airway hyperresponsiveness (Pijnenburg et al., 2005). On the other hand, researchers suggested increased ICS use in a F_{ENO}-guided patient group without clinical improvement compared to a conventional regime (Szefler et al., 2008). Lehtimäki et al. (2016) concluded in a systematic review that high F_{ENO} could predict ICS response in steroid-naïve asthma, and in ICS-treated asthmatics a low F_{ENO} predicts a low risk of exacerbation. However, they suggest the further need of conclusive information.

1.3 Other assessment methods of airway inflammation

Airway inflammation can be assessed by various techniques across a wide clinical spectrum, with mostly invasive methods and some non-invasive applications. These correlate with F_{ENO} to some degree, and previous researchers have studied the associations between them. F_{ENO} relates to tests which aim to measure a degree of eosinophilia. F_{ENO} correlates with peripheral blood eosinophils, induced sputum eosinophils, and eosinophils and eosinophilic cationic protein (ECP) obtained from bronchoalveolar lavage (BAL) (Badar et al., 2020; Berry et al., 2005; Lex et al., 2006; Rawy and Mansour, 2015; Riise et al., 2011). Later on, F_{ENO} was found to be associated with total IgE levels (Badar et al., 2020), and C reactive protein (CRP) (Rawy and Mansour, 2015). Juusela et al. (2013) found, in Finland, a correlation between BHR and F_{ENO} in non-smokers. A large population study in Norway (Henriksen et al., 2000) reported a high specificity of concomitant F_{ENO} and BHR for allergic asthma, and a correlation between F_{ENO} and BHR, as confirmed in a later study in children by Ciprandi et al. (2010), and individuals with allergic rhinitis (Cirillo et al., 2013).

1.4 Atopy and F_{ENO}

A follow-up study from a birth cohort in the Netherlands with measurements at young adult ages found higher F_{ENO} values in atopic asthmatics than non-atopic asthmatics (Van Asch et al., 2008). This study also found increased F_{ENO} in individuals with eczema. A general population study from Iceland reported an association between F_{ENO} and positive skin prick tests, as well as high total IgE and specific IgE (Thorhallsdottir et al., 2016). According to a study in a Peruvian general population, F_{ENO} had limited accuracy to identify atopy in adolescents, but had an increased accuracy for detecting atopic asthmatics. The same study found that a F_{ENO} >20 ppb predicted atopy with an accuracy of 68% (Romero et al., 2013). Positive skin prick tests without any respiratory symptoms do not increase F_{ENO} (Rouhos et al., 2008). In asthmatics with highly sensitised atopy, F_{ENO} was higher than in healthy controls (Ekroos et al., 2009), but asthmatics with no atopy or low sensitisation had similar F_{ENO} values. In atopic asthmatics, a F_{ENO} guided therapy achieved a reduction of exacerbations and improvement of symptoms without higher corticosteroid doses (Syk et al., 2013). Atopic individuals with allergic rhinitis had a higher F_{ENO} than non-atopic individuals (Kumar et al., 2013).

1.5 Asthma phenotypes and F_{ENO}

Asthma can be divided into different phenotypes, depending on its properties (e.g., biological pathways, clinical characteristics, response to drug types). Asthma phenotypes responsive to corticosteroids might be linked to high F_{ENO} values (Smith et al., 2005a). Most childhood asthma cases (early onset) are linked to the TH2 pathway with allergy and atopy involvement (Wenzel, 2012). These patients usually respond well to inhaled corticosteroids. An adult onset or late onset Th2-related phenotype does not respond to corticosteroids and is characterised by eosinophilia. These patients respond to a IL-5 antibody treatment (Wenzel, 2012). Another phenotype, which is Th2-associated, is exercise-induced asthma, with eosinophilia and mast-

cell activation.

Other phenotypes of asthma, without a Th2 component, are the very late onset asthma type and the neutrophilic asthma type. Very late onset asthma is obesity-related and more frequent in women. On the other hand, neutrophilic asthma is associated with smoking (Wenzel, 2012). Elevated F_{ENO} predicts, apart from corticosteroids, a response to biologicals (monoclonal antibodies), such as: omalizumab, mepolizumab, lebrikizumab and benralizumab (Ricciardolo and Silkoff, 2017), as explained in detail below. The Global Initiative for Asthma (2020) recommends adding biologicals in patients with exacerbations or poor asthma control, when already receiving a high dose of ICS-LABA, and indicates F_{ENO} as a predictor in cases of omalizumab and dupilumab use.

A therapy targeting IgE with omalizumab, a recombinant humanised monoclonal antibody, lowered the exacerbation rate and F_{ENO} level in severe asthmatics (Hanania et al., 2011). A high F_{ENO} before therapy with omalizumab may predict the response to the treatment (Hanania et al., 2013). Dupilumab, an IL-4 receptor α monoclonal antibody, reduces F_{ENO} and IgE, as well as exacerbations in patients with uncontrolled asthma, according to a randomised controlled trail (RCT) by Wenzel et al. (2013). In this study, patients belonging to a group with high baseline F_{ENO} (two high baseline F_{ENO} groups: $\geq$50 ppb and $\geq$25 ppb) benefited with a risk reduction of severe asthma exacerbations. Tezepelumab, a thymic stromal lymphopoietin IgG2 monoclonal antibody, lowers F_{ENO} and blood eosinophils after 4 week of treatment in adults with uncontrolled asthma compared to placebo, and patients receiving tezepelumab had fewer asthma exacerbations (Corren et al., 2017). In severe eosinophilic asthma, a humanised monoclonal IL-5 antibody, mepolizumab, lowers the risk of exacerbations, and blood eosinophil count and F_{ENO} both correlate with the response to mepolizumab, although F_{ENO} to a lesser extent (Pavord et al., 2012). A therapy with lebrikizumab, an IL-13 monoclonal antibody (humanised IgG4), reduced F_{ENO} in uncontrolled asthmatics compared to placebo in an RCT (Corren et al., 2011).

1.6 Asthma and F_{ENO} epidemiology in Northern Europe

Most previous studies found high prevalences of asthma in Northern European countries[§], but low prevalences were also found, depending on the country, year and methodology used. There seems to be an increase of asthma prevalence in Europe during the 20th century, probably attributable to a 'western' lifestyle and urban living (Gibson et al., 2013). In Nordic countries, for example, a study in Norway found an increase from 3.9% in 1972 to 9.3% in 1999 (Brogger et al., 2003). More recent studies have found a further increase of asthma prevalence, for example in Sweden (Backman et al., 2017), but the increase levelled off in 2016 in Finland (Hisinger-Mölkänen et al., 2019)(See Table 1.2 for a list of references reporting asthma prevalences in Nordic countries and Estonia). On the other hand, some Northern European countries like Estonia show a moderately lower prevalence of asthma. Observing the incidence of asthma, which indicates the new cases, in Table 1.3, there seems among the literature a very similar incidence rate in Nordic populations.

Table 1.2: Asthma prevalence in Nordic Countries and Estonia (Non-exhaustive list)

Country	Asthma prevalence (%)	Year(s)	Methodology	Reference
Sweden	8.4, 9.9, 10.9	1996,2006,2016	3 cross-sectional samples, postal questionnaire	(Backman et al., 2017)
Sweden	5.8–6.8	1994	Cross-sectional samples in multiple centres, postal questionnaire	(Burney, 1996)
Sweden	9.6, 11	2008,2016	2 cross-sectional samples, postal questionnaire	(Borna et al., 2019)
Finland	6.6, 10, 10.9	1996,2006,2016	3 cross-sectional samples, postal questionnaire	(Hisinger-Mölkänen et al., 2019)
Finland	7.2	1996	Postal questionnaire	(Pallasaho et al., 1999)
Finland	6	1995	Postal questionnaire	(Kotaniemi et al., 2001)
Finland	10.6	2006	Postal questionnaire	(Kainu et al., 2016)
Finland	4.2	2013	Register data	(Kankaanranta et al., 2017)
Estonia	2	1995–1998	Postal questionnaire	(Jannus-Pruljan, 2004)
Estonia	2.7	1995–1996	Postal questionnaire	(Meren et al., 2005)
Estonia	3.8	1997–2000	Clinical interview	(Meren et al., 2005)
Estonia	2	1994	Postal questionnaire	(Burney, 1996)
Iceland	3.4	1994	Postal questionnaire	(Burney, 1996)
Norway	4.3	1994	Postal questionnaire	(Burney, 1996)
Denmark	4	1994	Postal questionnaire	(Burney, 1996)

Few epidemiological studies in Nordic Countries have previously investigated F_{ENO} levels in the general population. Thorhallsdottir et al. (2016) found in Iceland, as part of The European Community Respiratory Health Survey (Burney, 1996) starting in 1996, a F_{ENO} mean (95% CI) of 16.2 (15.2–17.1) ppb, and reported a positive association between F_{ENO} and asthma,

[§]This literature overview focuses on Nordic Countries (Scandinavia) and Estonia, all belonging to Northern Europe, and the term 'Nordic populations' refers here to a population in Northern Europe

atopy, and high IgE. The Icelandic population has a relatively low prevalence of asthma. The Copenhagen General Population Study reported F_{ENO} (50 mL/s) median values stratified by clinical groups of airway disease in adults, and for example asthmatics had a median (25th and 75th percentile) value of 14 (9–21) ppb, and in comparison individuals without airflow limitation had a value of 13 (8–19) ppb (Çolak et al., 2018). This study also found an association between F_{ENO} and allergy, reversibility, asthmatic predisposition and some asthmatic symptoms, as well as reported a combined additive value of F_{ENO} and blood eosinophil tests to differentiate between diverse chronic obstructive diseases.

A general population study in Swedish adolescents found that F_{ENO} was associated with asthma and allergic symptoms, but not with spirometric forced expiratory volume in 1 s (FEV_1) (Nordvall et al., 2005). A follow-up of this cohort found higher F_{ENO} values in adolescents with allergic symptoms to cats or dogs compared to asymptomatic adolescents (Kalm-Stephens et al., 2019).

Table 1.3: Asthma incidence in Nordic Countries and Estonia (Non-exhaustive list).

Country or countries	Asthma incidence (/1000 per year)	Year(s)	Methodology	Reference
Sweden, Norway, Denmark, Iceland, Estonia	2.2	1999–2001	Longitudinal prospective	(Torén et al., 2004)
Sweden	3.2–4.5	1986–1996	Longitudinal prospective	(Lundbäck et al., 2001)
Sweden	2.4	1996 –2006	Longitudinal prospective	(Ekerljung et al., 2008)
Finland	2.5	2012–2013	Retrospective, register data	(Kankaanranta et al., 2017)
Norway	3.6	1997–2008	Longitudinal prospective	(Brumpton et al., 2016)

Du sehnst dich weit hinaus zu wandern,
Bereitest dich zum raschen Flug,
Dir selbst sei treu und treu den andern,
dann ist die Enge weit genug.

Far away you long to wander,
Prepare yourself and rush to fly,
Be true yourself and true the other',
Then narrowed paths far outlie.

J.W. von Goethe

2

Aims of the study

The aims of this study are in a broad sense to provide a deeper insight into the methodology of diagnosing asthma with clinical acquisition of F_{ENO}, and the epidemiology of asthma, F_{ENO}, and atopy in Northern European countries — Sweden, Finland and Estonia. The specific aims are synthesised in the following points and the Roman numerals indicate which publication addresses the aim:

- Elucidate the effect of carbonated water and tap water mouthwashes on F_{ENO} in relation to time (I).

- Investigate the effect of the mouthwashes — carbonated water and tap water — on F_{ENO} with multiple flow rates (II).
- Analyse the influence of a mouthwash procedure with carbonated water or tap water on extended respiratory NO parameters: Alveolar NO concentration (C_{ANO}), airway NO diffusion (D_{awNO}), and maximum airway NO flux ($\dot{J}_{awNO}$) (II).
- Develop a non-linear model for converting F_{ENO} obtained by different flows to the standard flow of 50 mL/s (III).
- Validate the conversion model in healthy paediatric populations and in healthy and diseased adult populations (cross-validation) (III).
- Apply the model in general population samples from Sweden, Finland, and Estonia by converting F_{ENO} values to a standard flow (IV).
- Examine the differences in standardised F_{ENO} values between the countries and study centres (IV).
- Assess the odds ratio for asthma in Sweden, Finland, and Estonia in relation to standardised F_{ENO}, atopy, smoking, gender, and study centre (IV).
- Compare the odds ratio for asthma in each study centre with the standardised F_{ENO} values and the prevalence of asthma, asthma symptoms, asthma medication, atopy and smoking (IV).
- Assess the hypothesis of a west–east gradient of allergic diseases in Northern European countries, based on F_{ENO} values (IV).

I warn you, if you bore me, I shall take my revenge.

J.R.R. Tolkien

3

Materials and methods

3.1 Publication I — Reduction of F_{ENO} by tap water and carbonated water mouthwashes: magnitude and time course

3.1.1 Participants

For this pilot study, we recruited 12 healthcare professionals with the following characteristics: healthy or asthmatic, non-smoking and aged 27–63 years. The participants were enroled without any further selection. They were instructed not to drink coffee 2 h before, and to not eat or drink 1 h before the study, nor to exercise prior to the measurements.

3.1.2 F_{ENO} measurements and mouthwashes

We performed F_{ENO} measurements according to the American Thoracic Society and European Respiratory Society (2005) guidelines, with an expiratory flow of 50 mL/s and NIOX VERO® equipment. We followed the manufacturer's instructions of the NIOX VERO® device. We obtained a baseline F_{ENO} mean from 2–4 single determinations. After obtaining a baseline F_{ENO}, the volunteers performed a 30 sec mouthwash and we immediately acquired a single F_{ENO} determination. Subsequent measurements were obtained every 2 min, totalling 10 measurements and 20 min. The volunteers repeated the F_{ENO} tests ca. 15 min later, but with another mouthwash solution. The mouthwashes employed were either tap water or carbonated water, with a mouthwash duration of 30 sec and 100 ml volume. The first volunteer had first a mouthwash with tap water, and then with carbonated water; the next volunteer had the opposite order of mouthwashes and the alternating order continued in that fashion until all 11 volunteers were tested. The F_{ENO} tests occurred at the Laboratory of Clinical Physiology at the University Hospital in Helsinki.

Mouthwashes: Chemical properties

The mouthwash solutions were tap water and carbonated water. The tap water utilised had a pH of 8.3 and the solutes and concentrations contained were:

$$ClO_2\ 0.5\ mg/L,\ K^+\ 1.4\ mg/L,\ Cl^-\ 5\ mg/L,\ Na^+\ 6\ mg/L,\ SO4^{2-}\ 10\ mg/L,\ Ca^{2+}\ 22\ mg/L$$

according to the water analyses from the Helsinki Region Environmental Services Authority (2017). The carbonated water's pH was 5.7–5.9 and contained $CaCl2,\ KHCO_3,\ MgCl_2,\ and\ NaHCO_3$ as characterised by the manufacturer of the bottled drink (HARTWALL VICHY ORIGINAL ®, Oy Hartwall Ab, Helsinki, Finland).

3.1.3 Statistics

Statistical analyses were made with IBM® SPSS® version 22 (IBM corporation, Armonk, NY, USA). The variables were normally distributed according to a Shapiro–Wilk test. We compared the differences in mean F_{ENO} with a general linear model (GLM) for repeated measurements. From the GLM we obtained estimated marginal mean F_{ENO} values in ppb with 95% CIs. The Figures for Publication I were obtained with GRAPHPAD® PRISM® version 5.04 (Graphpad Software, Inc., San Diego, CA, USA) and R (R Core Team, 2018).

3.2 Publication II — Influence of mouthwashes on extended exhaled nitric oxide (F_{ENO}) analysis

3.2.1 Participants

For this study, a total of 30 participants were recruited, including 21 healthcare workers and 9 patients. The healthcare workers who volunteered for the study were enroled without any further selection. The patients had asthmatic symptoms and had a referral for F_{ENO} testing. We gave the participants the following instructions: not to exercise prior the measurements, not to eat or drink 1 h before nor consume coffee 2 h before the study.

3.2.2 F_{ENO} measurements

We tested F_{ENO} at the Skin and Allergy Hospital, Helsinki or at the Finnish Institute of Occupational Health, Helsinki. We acquired the measurements from September 2016 until May 2017. Each participant was invited to perform measurements over two consecutive days. We followed the guidelines (American Thoracic Society and European Respiratory Society, 2005) for F_{ENO} measurements, apart from the experimental modifications of interest for this study: mouthwashes, multiple flows. The chemiluminescence NO analyser *CLD 88 sp* was incor-

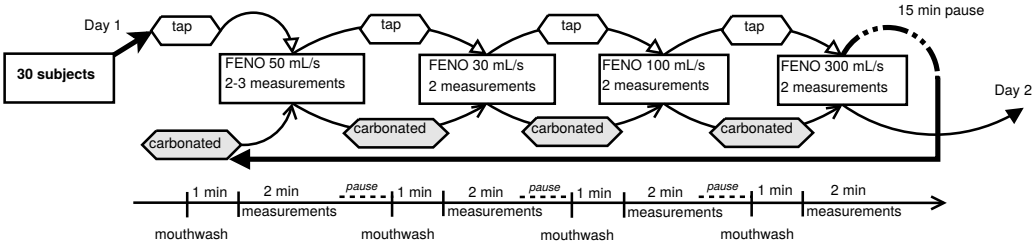


Figure 3.1: Illustration of the consecutive F_{ENO} determinations, the flows, mouthwashes and repetitions performed during one day. Tap: tap water mouthwash, carbonated: carbonated water mouthwash. *From: Lassmann-Klee et al. (2018a).*

porated in the EXHALIZER®'s device from EcoMedics AG (Dürnten, Switzerland) with a SPIROWARE® software. We calibrated the device following the operating instructions: use certified span gas (AGA Gas BV, Amsterdam, Netherlands). The inspired gas was NO-free (<5 ppb) air filtered with a zero-air device (DENOX 88 unit), with a maximum recorded fractional inspired NO of 3.1 ppb. Prior to measuring, we calibrated the ultrasonic flow sensor with a syringe (Hans Rudolph Inc., Shawnee, KS, USA).

Multiple flows

We measured F_{ENO} at four different expiratory flow rates $\dot{V}$ (30, 50, 100 and 300 mL/s), and the order of the measurements was kept constant for each participant, see flowchart in Figure 3.1

Mouthwashes

The participants rinsed the oral cavity at each measurement step for ca. 30–60 sec with 100 ml of tap water first and at the second array with 100 ml of carbonated water. See flowchart in Figure 3.1. All procedures were identical on the second day. We described the chemical properties of the mouthwashes above in Publication I.

3.2.3 Statistics

We performed the analyses with R (R Core Team, 2018) and IBM® SPSS® version 22 (IBM corporation, Armonk, NY, USA). We plotted the figures for Publication II with GRAPHPAD® PRISM® version 5.04 (Graphpad Software, Inc., San Diego, CA, USA) and R (R Core Team, 2018).

We compared differences in the F_{ENO} distributions with the Wilcoxon test for paired probes. We obtained a mean, F_{ENO} for each subject from 4 F_{ENO} measurements (2 each day) at flows of 30, 50, 100 and 300 mL/s, and after each mouthwash, respectively. We calculated the group mean (SD), median, and median absolute deviation (MAD). To ensure the accuracy of the measurements, we calculated the coefficient of variation c_v with 3.1 and defined a c_v <10% as acceptable.

$$c_v = \frac{\sigma}{\mu} \tag{3.1}$$

with μ as the mean and σ as the standard deviation. We compared the c_v values between mouthwashes with a Wilcoxon test, and all c_v results with a Friedman's two-way ANOVA.

Extended F_{ENO} analyses

We obtained C_{ANO}, D_{awNO}, and $\dot{J}_{awNO}$ by applying equation 3.2, as well with the algorithm developed by Högman and Meriläinen (2007) (HMA).

$$logF_{ENO} = log(\frac{\dot{J}_{awNO}}{D_{awNO}} + (C_{ANO} - \frac{\dot{J}_{awNO}}{D_{awNO}})^{\frac{-D_{awNO}}{\dot{V}}}) + \varepsilon \tag{3.2}$$

Equation 3.2 is a nonlinear logarithmic transformation developed by Eckel et al. (2014) with starting estimated values of the quadratic T transformation (a second order approximation). The HMA necessitates F_{ENO} values obtained from three different flows $\dot{V}$: 30 mL/s, 100 mL/s and 300 mL/s.

We compared C_{ANO}, D_{awNO}, and $\dot{J}_{\mathrm{awNO}}$ values between mouthwashes with a Wilcoxon test for paired probes.

3.3 Publication III — Converting F_{ENO} from different flows to standard flow F_{ENO}

3.3.1 Participants

The participants for this study were identical to Publication II, with the difference that additional measurements were acquired (see below) from 10 healthcare workers, with the sole inclusion criteria of working at the Skin and Allergy Hospital, Helsinki and that our results were validated with 5 different datasets from the Tampere University Hospital, see Table 3.1.

3.3.2 F_{ENO} measurements

We measured F_{ENO} as in Publication II, but additionally acquired a third day F_{ENO} of determinations without a mouthwash at 50 mL/s, 30 mL/s, 100 mL/s, and 300 mL/s in the 10 healthcare workers.

Table 3.1: Datasets employed in validating the conversion model.

n	Age	Diagnoses	Reference
69	adults	healthy	Lehtimäki et al. 2010 a, Lehtimäki et al. 2010 b
66	children	healthy	Sepponen et al. 2008
74	adults	COPD	Lehtimäki et al. 2010 a
40	adults	steroid-naïve asthma	Lehtimäki et al. 2001
17	all	untreated alveolitis	Lehtimäki et al. 2001

3.3.3 Statistics

We analysed the data with *R* (R Core Team, 2018) and the frontend RStudio® (RStudio Team, 2020), as well as plotted the figures with the same statistical software. We obtained from each flow level (i.e., 50 mL/s, 30 mL/s, 100 mL/s, and 300 mL/s) the arithmetic mean F_{ENO} for each individual. We plotted these mean F_{ENO} values against the expiratory flow rate $\dot{V}$ in a double logarithmic scale. We applied a non-linear regression. From the non-linear regression, we obtained a slope and intercept. We analysed the regression line to develop our conversion model. We refined our model through acquiring a non-linear least squares estimation of the non-linear model parameters. We employed this final model to obtain $\hat{F}_{\text{ENO}}$ estimates from F_{ENO} determinations measured at other flow rates (see below). We controlled the raw data for outliers with the absolute deviation around the median, with three deviations as the cut-point (Leys et al., 2013). Additionally, we analysed with a linear regression (GLM) the F_{ENO} values measured at 50 mL/s after both mouthwashes — tap water and carbonated water — to evaluate their relationship. This further supplies an additional equation for converting values of F_{ENO} (at flow level 50 mL/s) between mouthwashes.

F_{ENO} Validation

We converted our measured F_{ENO} values at the flows 30, 100 and 300 mL/s to estimated $\hat{F}_{\text{ENO}}$ values at 50 mL/s, the standard flow rate. Subsequently, we calculated the agreement between the estimated $\hat{F}_{\text{ENO}}$ and the measured F_{ENO} with a method described by Bland and Altman (2010). We additionally tested the linearity of values with Spearman's correlation coefficient ϱ (rho).

This study included an external validation of our conversion model in the 5 datasets presented in Table 3.1. We tested the conversion model through estimation of $\hat{F}_{\text{ENO}}$ at 50 mL/s or 40 mL/s (depending on the datasets) from F_{ENO} values measured at 100 mL/s. We further

compared the estimates with obtained values at the same flow and calculated the individual differences of F_{ENO}. We then obtained the mean of the differences (bias) with its 1.96 standard deviations (95% limits of agreement).

3.4 Publication IV — Parallel gradients in F_{ENO} and in the prevalences of asthma and atopy in adult general populations of Sweden, Finland and Estonia — A Nordic EpiLung study

3.4.1 Introduction to the study populations

As part of a cross-sectional study, the first step consisted of a postal questionnaire on respiratory symptoms and diseases in random samples of general populations in 5 areas from Sweden, Finland and Estonia during the years 1995–1996.

The study centres (see Figure 3.2) — Stockholm, Örebro, Helsinki, Narva, Saaremaa — continued the cross-sectional study with a structured clinical interview, F_{ENO} and skin prick tests, and lung function measurements, which formally constitutes the scope of the present study (Publication IV).

Participants

We sent the above-mentioned postal questionnaire to 34 951 adults randomised into 10-year age-and-gender-distributed cohorts from the Population Register Centres in Stockholm, Örebro, Helsinki, Narva, and Saaremaa. See the flowchart in Figure 3.3 for detailed steps and numbers from the postal questionnaire, clinical interview and tests.

We obtained completed questionnaires from 27 697 (79% of invited): 5 754 (72%) in Stockholm, 6 784 (84%) in Örebro, 6 062 (76%) in Helsinki, 4 325 (78%) in Narva, and 4 808 (89%) in Saaremaa. We invited a random sample stratified by age and gender to participate in the clinical examinations, totalling 4 944 individuals and representing 18% of those who participated in the postal questionnaire (responders). 54% of the invited participated, totalling 2 658 volunteers: 443 (37% of locally invited) in Stockholm, 719 (60%) in Örebro, 643 (54%) in Helsinki, 402 (60%) in Narva, and 451 (67%) in Saaremaa.

Figure 3.2: Map of the participating centres in Finland, Estonia and Sweden (FinEsS). *From Lassmann-Klee et al. (2020).*

We randomised the 2 658 volunteers from the clinical interview. We measured F_{ENO} and performed skin prick tests on 1 669 random participants in the years 1997–2003. After excluding volunteers with technically invalid measurements and F_{ENO} under 20 or over 60 years, we included 1 498 for this study. 92% of these volunteers had valid SPTs.

3.4.2 Questionnaire

We based the postal questionnaire on a questionnaire developed by the OLIN (Obstructive Lung Diseases in Northern Sweden) research project, a modified version of the British Medical Research Council and Tucson questionnaires (Pallasaho et al., 1999). For the clinical interview, we modified the postal questionnaire with additional questions of symptoms, medication, and possible risk factors for obstructive airway diseases and allergy. Professional interpreters, with expertise in the study topic, translated the questionnaire in all native languages

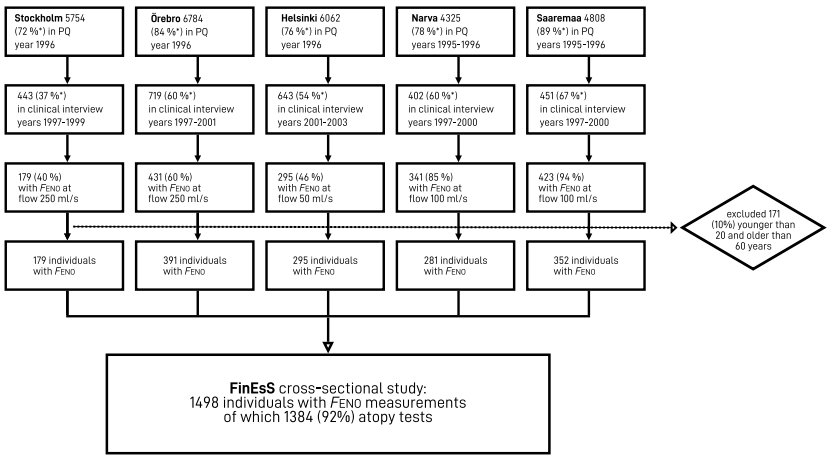


Figure 3.3: Flowchart of the population selection in Finland, Estonia and Sweden (FinEsS). Presented as n, (%*) percentage of invited, or (%) percentage of total. Postal questionnaire (PQ), fractional exhaled nitric oxide (F_{ENO}). *From Lassmann-Klee et al. (2020).*

(Finnish, Swedish, Estonian, and Russian). We chose 11 questions from the clinical interview that referred to self-reported or diagnosed obstructive respiratory diseases, asthma symptoms and medication, smoking, and self-reported allergic rhinitis or conjunctivitis.

Definitions

We created the following definitions used in the study through the affirmative answers to the respective question in the interview.

- Asthma: have you ever had asthma?
- Asthma diagnosis: Have you been diagnosed with asthma by a physician?
- Childhood asthma: Have you had asthma during childhood or had a wheeze during breathing in early childhood?
- Chronic obstructive pulmonary disease (COPD) diagnosis: Have you been diagnosed with chronic bronchitis or emphysema by a physician?

- Current smoker: Are you a current smoker?
- Allergic rhinitis or conjunctivitis (ARC): Have you (currently) or have you had hay fever (allergic rhinitis) or allergic eye inflammation?
- Asthma symptoms during the last year: Have you had any asthma symptoms during the last 12 months?
- Nightly asthma symptoms last year: Have you ever been woken up during the night or early morning by an attack of shortness of breath with wheezing? If yes, has this happened during the last 12 months?
- Asthma medication during the last year: Have you used any asthma medicines during the last 12 months?
- Inhaled corticosteroids (ICS) last year: Have you used inhaled corticosteroids during the last 12 months (List of national commercial names)?
- Short-acting β-agonists (SABA) last year: Have you used inhaled short-acting β-agonists during the last 12 months (List of national commercial names)?

3.4.3 F_{ENO} MEASUREMENTS

We measured F_{ENO} following the ATS and ERS guidelines (American Thoracic Society and European Respiratory Society, 2005), with the sole exception that the centres employed different expiratory flows. The F_{ENO} device was a chemiluminescence nitric oxide (NO) analyser, Sievers 270B (Boulder, CO, USA) and was calibrated daily according to the manufacturers instructions (two-point calibration). We used NO-free synthetic air. We used resistors for controlling the target expiratory flow range (Hans Rudolph Inc., Shawnee, KS, USA) with an additional display for visual control. The resistor also served the purpose of closing the soft palate to avoid nasal NO contamination. We did not use nose-clips for the same reason. The exhalation time

was ≥6 s (usually 10 s). We obtained an arithmetic mean F_{ENO} from three consecutive end-of-exhalation plateau determinations. We performed mouthwashes prior to the F_{ENO} measurements to reduce oral contamination (see Publication I and II): in Helsinki with carbonated water, and in other centres with tap water. We choose a cut-off value of ≥25 ppb as a positive (intermediate) F_{ENO} as suggested by the ATS (Dweik et al., 2011) and ERS guidelines (Horváth et al., 2017).

F_{ENO} flows

The study centres applied different expiratory flow rates when measuring F_{ENO}. The expiratory flow rates were: 250 mL/s in Stockholm and Örebro, 50 mL/s in Helsinki, and 100 mL/s in Narva and Saaremaa. We converted all F_{ENO} values to the standard flow rate — 50 mL/s — using the previously model developed (Publication III) for standardisation of F_{ENO} flows and mouthwashes.

3.4.4 Skin prick tests

We performed SPTs on a total of 1 334 volunteers (92% of the participants). We tested for the following allergens as single tests on one forearm with commercial extracts:
Canis lupus familiaris (dog), Felis catus (cat), Equus caballus (horse), Phleum pratense (timothy-grass), Betulaceae sp. (birch), Artemisia sp. (mugwort), Alternaria alternata, Cladosporium herbarum, Dermatophagoides pteronyssinus, Dermatophagoides farinae. Additionally, in all centres apart from Stockholm, we tested for: *Lepidoglyphus destructor (storage mite), Acarus siro (storage mite), Bos taurus (cow), Blatella germanica (cockroach)* and *latex*. A volunteer was classified as atopic if at least one allergen SPT was positive, defined as a ≥3 mm reaction. We rejected reactions with dermographism. Previous researchers described this testing protocol (Pallasaho et al., 2006), which complies with recent European guidelines (Heinzerling et al., 2013).

3.4.5 Spirometry

We performed spirometry tests and acquired FEV_1 — the highest value of three acceptable determinations — in the centres with the following devices: *Ohio spirometer* (Stockholm), *Volugraph 2000* and *Vitalograph* (Örebro), *SensorMedix Vmax22* (Helsinki) and *Mijnhardt Vicatest 5* (Narva and Saaremaa).

3.4.6 Statistics

We performed the statistical analyses with *R* (R Core Team, 2018) and RStudio® (RStudio Team, 2020). We plotted our figures with the same software. F_{ENO} was not normally distributed in the general population, so we tested F_{ENO} between the countries and centres with a Kruskal–Wallis test and applied a post-hoc test (Dunn's test for multiple comparison) with Holm's adjustment. When comparing two-group variables, we compared the F_{ENO} distributions with a Wilcoxon–Mann–Whitney test for independent groups. We calculated two-by-two tables and corresponding raw odds ratios (OR) and the 95% confidence intervals (CI) using the method number ten by Newcombe (1998). We calculated p values for the CIs with Pearson's χ^2 test with Yates' continuity correction. Additionally, we estimated adjusted ORs by binary logistic regression. Within the binary logistic regression model, we tested the multiple parameters of the study centre variable with a Wald χ^2 test. We selected the best fitting models with the Akaike information criterion (AIC). We obtained a minimum sample size of n = 1232 for binary logistic regression when considering an OR of 1.5 for an event and a prevalence of 4%, according to Hsieh et al. (1998).

3.5 Ethics

The pertaining local ethical committees (Helsinki University Hospital Medical Ethical Committee (99/13/03/00/15 and HUS/3332/2017), the Tallinn Ethics Committee in Estonia and the

Swedish Ethical Review Authority) approved the studies (99/13/03/00/15 and HUS/3332/2017). Before participating, all participants signed a written informed consent.

Si hortum in bibliotheca habes, deerit nihil.

If you have a garden and a library,

you have everything you need.

Marcus Tullius Cicero

4

Results

4.1 Publication I and II — Mouthwashes' effect on F_{ENO}

This section summarises the results obtained after employing two mouthwashes, tap water and carbonated water, i.e., the mouthwashes' effect on F_{ENO}, first F_{ENO} measured at the standard flow $\dot{V}$ of 50 mL/s and second, measured at different flows 30, 100 and 300 mL/s.

4.1.1 Effect of mouthwashes on F_{ENO} at flow $\dot{V}$ 50 mL/s

We found an immediate effect of both mouthwashes — tap water and carbonated water — on F_{ENO} measured at flow $\dot{V}$=50 mL/s, both mouthwashes independently decreasing F_{ENO} at the individual level, see Table 4.1. This effect prevailed for both mouthwashes for 2 min. Individual F_{ENO} values at the baseline, immediately after the mouthwash and after 2 min are visualised in Figure 4.1. This Figure indicates individual differences on the magnitude of mouthwashes' effect.

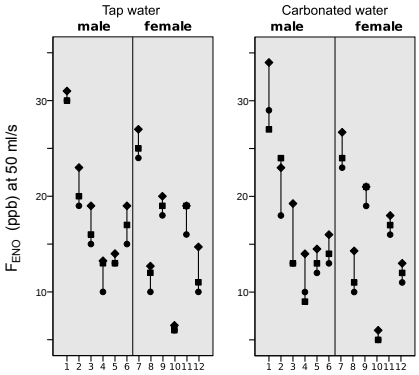


Figure 4.1: F_{ENO} (ppb) after tap water mouthwash (left side) and carbonated water mouthwash (right side). Individual F_{ENO} values (n=12) separated by gender, during baseline (♦ rhombi), immediately after mouthwash (■squares), and after 2 min (● circles). *From:* *Lassmann-Klee et al. (2018b).*

4.1.2 Time course

After the immediate lowering of F_{ENO} through the tap water mouthwash, F_{ENO} returned near the baseline level during the consecutive measurements from 4–20 min (see Figure 4.1.2), where statistical tests showed no difference compared to the baseline, apart from the measurement at 14 min, where F_{ENO} was higher: 19.2 ppb, 95% CI [14.0; 24.4].

After the initial 2 min, the carbonated water mouthwash lowered F_{ENO} significantly during the interval 2–12 min ($p<0.05$), compared to the baseline. At minute 14, the F_{ENO} level increased, and remained at a high level during further measurements (Figure 4.2).

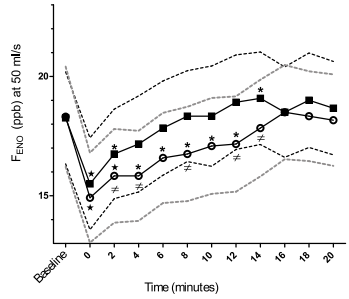


Figure 4.2: Arithmetic mean F_{ENO} (ppb) after tap water mouthwash (rectangles) and carbonated water mouthwash (circles) in relation to time (min) and baseline. Baseline F_{ENO} obtained prior to mouthwash. Dotted lines represent the 95% CI (grey dotted line represents the 95% CI for carbonated water and black dotted line represents the 95% CI for tap water). Tested with GLM for repeated measures. Legend: ★ p < .001 compared with baseline, *p < .05 compared with baseline, ≠ p < .05 pairwise comparison. *From:* *Lassmann-Klee et al. (2018b).*

Pairwise comparisons

We found a difference of the estimated marginal mean F_{ENO} between mouthwashes (p=0.008). F_{ENO} was lower after mouthwash with carbonated water — 17.0 ppb, 95% CI [12.0; 22.1] — than after the mouthwash with tap water — 18.0 ppb, 95% CI [12.9; 23.1]. Pairwise comparisons between the mouthwashes showed no difference in F_{ENO} immediately after the mouthwash procedure. Nevertheless, we found lower F_{ENO} values for the carbonated mouthwash, compared to the tap water mouthwash at determinations acquired at time points 2, 4, 8, 12, and 14 mins.

Table 4.1: Immediate effect of mouthwashes on F_{ENO} (ppb) and after 2 min compared to baseline, estimated marginal means. *Legend*: * p<0.001 and † p<0.05 for within-subject contrasts.

Mouthwash	Baseline	95% CI	Immediate	95% CI	2 min	95% CI
tap water	18.1	13.1–23.2	15.7*	10.7–20.7	17†	12.1–21.9
carbonated water	17.9	12.9–22.9	14.6*	10.1–19.2	15.1†	10.7–19.5

4.1.3 Mouthwashes' effect on F_{ENO} with different expiratory flows

Table 4.2: F_{ENO} (ppb) mean at every flow rate and mouthwash, either with tap water or carbonated water. *Legend*: † Relative decrease of the mean, ‡ Confidence of variation, mean (SD). *Modified from:* Lassmann-Klee et al. (2018a)

F_{ENO} (ppb)	Mean(SD)	Difference †	c_v ‡
Flow 30 mL/s, tap water	39.21(57.04)	-4.6%	8.6 (4.5)
Flow 30 mL/s, carbonated water	37.40(59.28)		7.3 (5.0)
Flow 50 mL/s, tap water	27.27(40.19)	-6.4%	8.4 (6.2)
Flow 50 mL/s, carbonated water	25.51(38.01)		6.6 (3.9)
Flow 100 mL/s, tap water	15.41(21.58)	-4.4%	7.0 (6.6)
Flow 100 mL/s, carbonated water	14.74(21.72)		6.1 (4.0)
Flow 300 mL/s, tap water	6.03(7.29)	-4.2%	6.9(4.1)
Flow 300 mL/s, carbonated water	5.77(7.45)		8.0 (5.3)

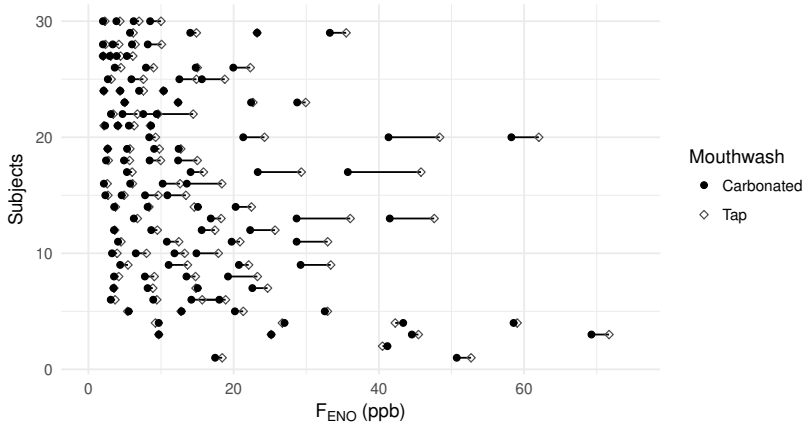


Figure 4.4: F_{ENO} values from studied volunteers, measured at multiple expiratory flow levels. Ordinate represents the individuals (n = 30), abscissa represents F_{ENO} (ppb) at multiple expiratory flow levels after: carbonated water mouthwash (black dots) and tap water mouthwash (grey rhombi). Abscissa truncated at 75 ppb. Subjects 1 and 2 had higher F_{ENO} values (not shown). The missing higher values (tap water/carbonated) for subject 1 are: 128/128, 85/79; and for subject 2 are: 311/322, 222/208, 117/117. *From:* Lassmann-Klee et al. (*2018a*).

We found a lower F_{ENO} acquired after a mouthwash with carbonated water compared to a tap water mouthwash at every expiratory flow rate (30, 50, 100, and 300 mL/s), as shown in Figure 4.3. The differences in mean F_{ENO} between mouthwashes varied from -4.2 to -6.4%. The coefficient of variation c_v stayed low, under 10%, showing an exact procedure with minimal variation between repeated measurements (see Table 4.2). We observed lower F_{ENO} values after a carbonated water mouthwash, but there were individual variations, depending on the overall F_{ENO} level and expiratory flow rate, e.g., certain participants with overall high F_{ENO} values were prone to show a salient decrease in F_{ENO} after the carbonated water mouthwash as observed in participants nr. 13, 17, and 20 (see Figure 4.4 with individual F_{ENO} values from each participant).

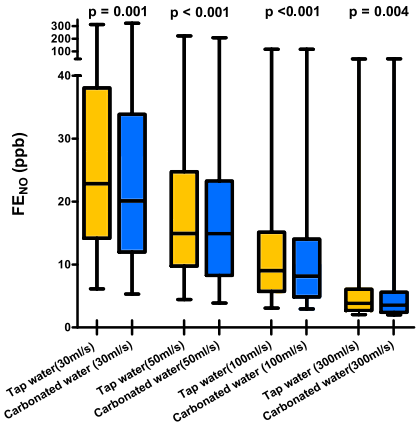


Figure 4.3: F_{ENO} at multiple expiratory flow levels after different mouthwashes. *p* values from Wilcoxon signed-rank test. *Modified from:* Lassmann-Klee et al. (*2018a*)

Table 4.3: Median (median absolute deviation) values of maximum airway NO flux ($\dot{J}_{awNO}$), alveolar NO concentration (C_{ANO}), and airway NO diffusion (D_{awNO}), after carbonated water or tap water mouthwashes. *p* values obtained with Wilcoxon signed–ranks tests. *Legend*: † Calculated with Högman and Meriläinen algorithm. *From:* Lassmann-Klee et al. (2018a).

	$\dot{J}_{awNO}(\frac{pL}{s})$	p	C_{ANO} (ppb)	p	D_{awNO} $(\frac{pL}{s \cdot ppb})$	p
Tap water	836.36 (457.58)	0.015	0.88 (0.48)	0.715	14.19 (7.52)	0.299
Carbonated water	829.97 (443.12)		0.92 (0.33)		18.60 (3.87)	
Tap water †	796.88 (449.86)	<0.001	1.00 (0.39)	0.598	15.63 (6.95)	0.871
Carbonated water †	724.96 (392.60)		1.04 (0.30)		15.28 (3.67)	

Influence of mouthwashes on extended analysis of exhaled nitric oxide

We found that when comparing the carbonated and tap water mouthwash, the median values of maximum airway NO flux ($\dot{J}_{awNO}$) were lower after the carbonated water mouthwash. The median values for $\dot{J}_{awNO}$ were lower with both algorithms employed (Högman and Meriläinen, 2007; Eckel et al., 2014). On the other hand, alveolar NO concentration (C_{ANO}) and airway NO diffusion (D_{awNO}) were similar after both mouthwashes.

4.2 Publication III — Converting F_{ENO} of different flows to standard flow F_{ENO}

Within this section we present a novel model for converting F_{ENO} values obtained at different expiratory flows and assess its validity in five external populations (datasets presented in Table 3.1). We also show a conversion equation for estimating values between mouthwashes at the standard expiratory flow of 50 mL/s.

4.2.1 Conversion model

We performed a non-linear regression and acquired non-linear least-squares parameter estimates, which resulted in slope values for the different mouthwash settings: for carbonated water -0.8416 SE(0.3192), for tap water -0.84 SE(0.2989), and without mouthwash a slope of

-0.83111 SE(0.05424). The equation for the model can be defined as:

$$\hat{F}_{ENO} = k \cdot \dot{V}^{-0.83111} \tag{4.1}$$

with $\hat{F}_{ENO}$ as estimated F_{ENO}, constant k, and expiratory flow rate $\dot{V}$. We plotted the model with equation 4.1 using measured F_{ENO} and $\dot{V}$, and obtained values for k (Figure 4.5). The equation 4.1 and Figure 4.5 exemplify the model without a mouthwash, and interchanging the slope with aforementioned values results in similar equations and plots. The equation can be formulated in a general way:

$$\hat{F}_{ENO} = \dot{J}_{awNO} \cdot \dot{V}^{-C_{ANO}} \tag{4.2}$$

with $\dot{J}_{awNO}$ as the maximum airway NO flux and C_{ANO} the alveolar NO concentration.

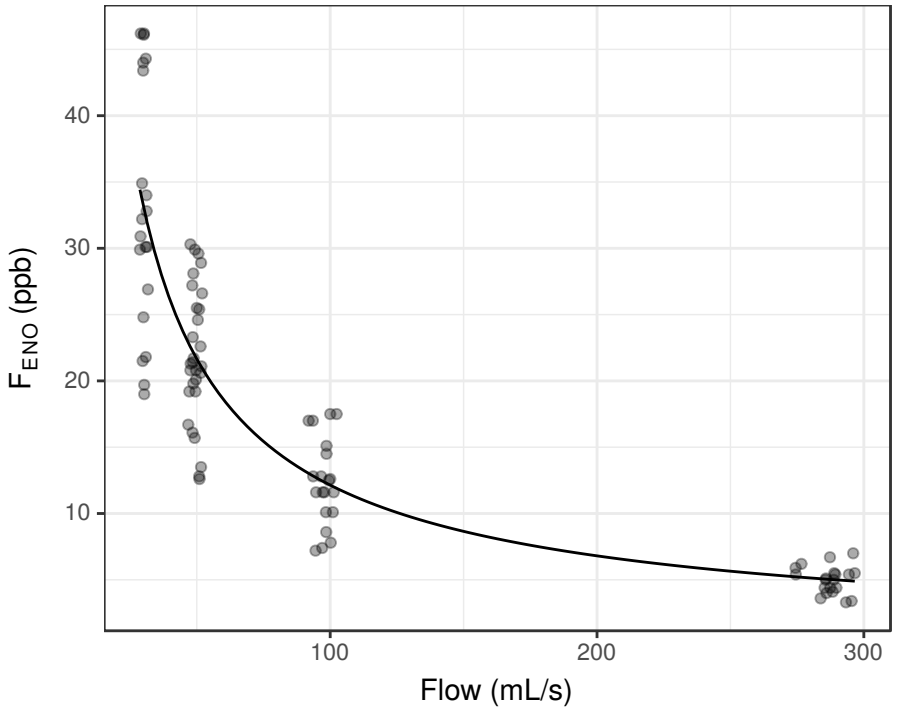


Figure 4.5: F_{ENO} as a function of expiratory flow (without mouthwash), n = 10. *Modifed from: Lassmann-Klee et al. (2019)*

Table 4.4: Bland–Altman statistics with bias †, levels of agreement and standard deviation (SD) of the differences between estimated $\hat{F}_{ENO}$ from 100 mL/s (Eq. 4.1) and measured F_{ENO} at 50 or 40 mL/s. *Legend*: † average of the differences. *From* Lassmann-Klee et al. (2019)

		level of agreement		
Population	bias†	lower	upper	SD
Mixed healthy and asthmatic adults	-0.28	-7.42	6.86	3.64
Healthy adults	-0.44	-3.87	2.98	1.74
Asthmatic	-1.68	-11.36	7.99	4.94
Healthy children	0.27	-1.94	2.48	1.13
COPD	-1.16	-11.46	9.13	5.25
Alveolitis	1.47	-8.28	11.22	4.98

4.2.2 Model validation

We employed the obtained equation 4.1 to estimate $\hat{F}_{ENO}$ values interpolated for a flow level of 50 mL/s from data measured at 100 mL/s. We used the method by Bland and Altman (2010) to evaluate the differences between the estimated — with the model — and measured F_{ENO}. We also calculated the mean (SD) differences, and levels of agreement (1.96 standard deviations) between these estimated and measured values for five external populations (see datasets in Table 3.1). The average difference varied between the populations, with small differences in healthy and mixed populations, and highest in asthmatics. Additionally, the limits of agreement were similar, with narrow limits of agreement in healthy populations and broader in populations with diseases. These results are summarised in Table 4.4. Visualising the distribution of the average difference between the estimated $\hat{F}_{ENO}$ and measured F_{ENO} with a density plot (Fig. 4.6) shows curves with high density near zero ppb, apart from the curves from the asthmatic adult and the alveolitis populations, which are slightly skewed.

4.2.3 Effect on F_{ENO} at flow $\dot{V}$ 50 mL/s

Performing a linear regression of F_{ENO} as response variable at flow $\dot{V}$ 50 mL/s

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i \tag{4.3}$$

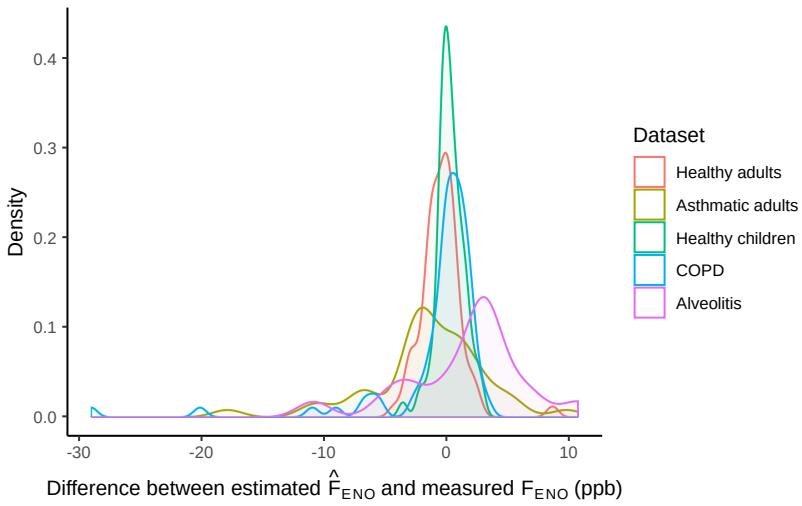


Figure 4.6: F_{ENO} density of mean differences between estimated $\hat{F}_{ENO}$ from 100 mL/s and measured F_{ENO} at 50 or 40 mL/s in all studied populations. *From:* *Lassmann-Klee et al. (2019).*

with the carbonated water mouthwash as the reference resulted in the following slope and intercept for tap water:

$$Y_i = 0.354 + 1.055X_i + \varepsilon_i \tag{4.4}$$

4.3 Publication IV — Parallel gradients in F_{ENO} and in the prevalences of asthma and atopy in adult general populations of Sweden, Finland and Estonia — A Nordic EpiLung study

This epidemiological study of F_{ENO}, asthma and atopy in Northern European Countries included a total of 1498 individuals from the five study centres. The selected populations were mutually similar, with comparable age, height, weight, body mass index (BMI) and FEV_1 results (Table 4.5).

Table 4.5: Anthropometric data and forced expiratory volume in one second (FEV_1) in adult general populations of Stockholm, Örebro, Helsinki, Narva, and Saaremaa. *From Lassmann-Klee et al. (2020)*

		Total	Stockholm	Örebro	Helsinki	Narva	Saaremaa
	n	1498	179	391	295	281	352
Gender	f/m	824/674	99/80	199/192	174/121	162/119	190/162
	%	55/45	55/45	51/49	59/41	58/42	54/46
Age (years)	mean (sd)	40.9 (10.9)	39.3 (10.0)	42.1 (10.8)	40.9 (10.2)	40.2 (11.6)	41.0 (11.4)
Height (cm)	mean (sd)	171.1 (9.3)	172.9 (9.6)	172.2 (9.2)	170.0 (9.3)	168.6 (8.7)	171.7 (9.3)
Weight (kg)	mean (sd)	75.3 (14.7)	72.7 (13.5)	75.5 (13.8)	75.2 (16.2)	73.7 (13.5)	77.6 (15.7)
BMI (kg/m²)	mean (sd)	25.7 (4.5)	24.2 (3.5)	25.4 (3.9)	26.0 (5.0)	25.9 (4.4)	26.3 (5.0)
FEV_1 (L)	mean (sd)	3.4 (0.9)	3.7 (0.8)	3.2 (0.8)	3.3 (0.9)	3.4 (0.9)	3.6 (0.9)

4.3.1 F_{ENO} in the Nordic populations

We found a median (interquartile range) of F_{ENO} in the total sample (n=1498) of 14.0 (10.5) ppb. The median (IQR) was 15.5 (9.3) ppb in Sweden, in Finland 15.4 (13.6) ppb and in Estonia 12.5 (9.6) ppb, with differences in their distributions — Kruskal–Wallis test, p<0.001. Performing a post-hoc analysis showed lower median F_{ENO} in Estonia and the other countries (p<0.001). Similarly, the median F_{ENO} was dissimilar in the five centres (p<0.001) (Figure 4.7). The centres in Estonia had a lower median F_{ENO} (p<0.001). Specifically, we found differences between Narva and Stockholm (p=0.001), Narva and Helsinki (p<0.001), and Narva and Örebro (p<0.001); as well as differences between Saaremaa and Örebro (p<0.001), and Saaremaa and Helsinki (p=0.002).

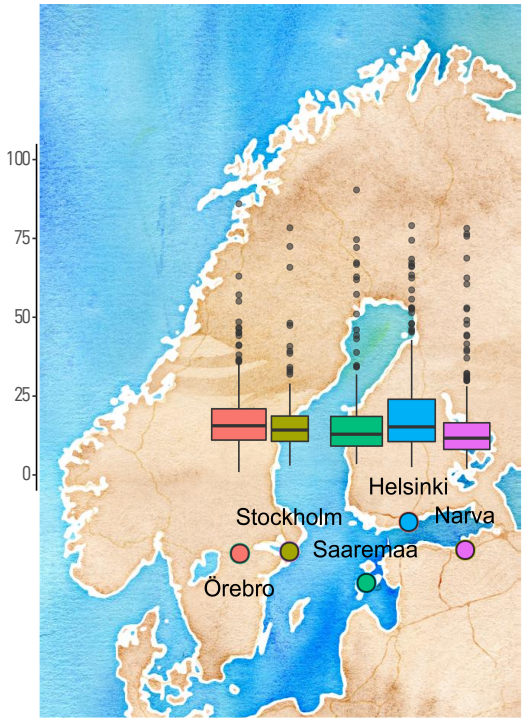


Figure 4.7: F_{ENO} boxplots with median and interquartile range in participating centres in Finland, Estonia and Sweden as a geographical map. *From Lassmann-Klee et al. (2020). Map tiles by Stamen Design (CC BY 3.0) and OpenStreetMap (ODbL).*

4.3.2 Prevalence of respiratory symptoms and diagnoses

As highlighted in the previous section, F_{ENO} varied significantly between the centres. Likewise, the prevalences of the respiratory symptoms and diagnoses varied (Table 4.6). We found the highest prevalence of asthma in Stockholm (13%), as well as asthma diagnosis (11%), and asthma symptoms in the last year (13%), and consequently the highest prevalence of asthma drug use (ICS 9%, SABA 10%). On the other hand, Saaremaa had the lowest prevalences of asthma (3%) and asthma diagnosis (2%), as well symptoms and drug use. Narva showed a high COPD prevalence (18%) and also current smokers (41%). The population in Helsinki had a large prevalence of allergic diseases: atopy (49%), and allergic rhinitis or conjunctivitis (ARC) (40%).

Table 4.6: Median F_{ENO} (interquartile range (IQR)) and prevalence of asthma, respiratory symptoms within the last year, and atopy in Stockholm, Örebro, Helsinki, Narva, and Saaremaa. *From Lassmann-Klee et al. (2020)*

		Stockholm	Örebro	Helsinki	Narva	Saaremaa	p
F_{ENO} median (IQR)		14.4 (8.0)	15.7 (10.1)	15.4 (13.6)	11.8 (8.6)	13.1 (9.5)	<0.001*
		%	%	%	%	%	
Ever asthma	yes	12.8	8.5	9.1	6.3	2.9	<0.001
Asthma by MD†	yes	11.4	7.8	7.2	5.4	2.3	<0.001
Asthma in childhood	yes	8	8.6	7.3	1.8	6.4	<0.01
Asthma symptoms	yes	13.4	9.2	12.5	6.1	2.6	<0.001
Nightly symptoms	yes	7.3	4.9	1	6.4	4.3	<0.01
Asthma medication	yes	12.3	6.9	9.2	4.6	2.3	<0.001
ICS‡ used	yes	8.9	4.1	5.1	1.1	0.6	<0.001
SABA§ used	yes	10.1	5.9	4.4	3.6	2.3	<0.01
SPT¶	positive	21.2	33.5	48.5	37.7	25.8	<0.001
Allergic rhinitis	yes	33	39.4	40	32.4	17.6	<0.001
Current smoking	yes	37.2	22	37	40.5	34.1	<0.001
COPD‖ by MD	yes	2.9	1.31	2.8	17.8	9.2	<0.001

*p values calculated with Kruskal-Wallis test, otherwise with Pearson's χ^2, †medical doctor, ‡inhaled corticosteroids, §short-acting β_2-agonist, ¶skin prick tests, ‖chronic obstructive pulmonary disease

4.3.3 Comparisons for asthma likelihood between Nordic populations

When estimating crude odds ratios for asthma probabilities between countries, we found a higher likelihood of asthma in Sweden — OR 2.39 (1.48–3.85) — and Finland — 2.0 (1.12–

3.58) — than in Estonia. Sweden and Finland had a similar likelihood of having asthma, with OR 1.19 (0.71–2.0).

4.3.4 Asthma and F_{ENO}

We analysed the association of self-reported asthma and $F_{ENO} \geq 25$ with a binary logistic regression model (Eq. 4.5), and found individuals with results of $F_{ENO} \geq 25$ were more likely asthmatics, with OR 3.91 (2.39–6.32). This model was adjusted for smoking, gender, skin prick test result and study centre. The variable study centre was associated with asthma ($p<0.01$), and we found different associations depending on the study centre: compared with Saaremaa, individuals were more likely to have asthma in Helsinki OR 2.40 (1.04–6.02), Narva OR 2.45 (1.05–6.19), Örebro OR 3.38 (1.59–8.09), and Stockholm OR 5.54 (2.18–14.79). We found that asthma was associated with a positive SPT result, OR 3.19 (2.02–5.11). The covariates smoking OR 0.87 (0.50–1.46) and gender OR 0.81 (0.51–1.27) were not associated with asthma. These results are visualised in Figure 4.8.

$$logit(\pi_i) = \beta_1 + \beta_2 \cdot FENO_i + \beta_3 \cdot Smoking_i + \beta_4 \cdot Centre_i + \beta_5 \cdot Gender_i + \beta_6 \cdot Skin\ prick\ test_i \tag{4.5}$$

F_{ENO} and asthma in relation to smoking and atopy

The participants with asthma in the whole sample (n=1498) had a higher F_{ENO} — 27 ppb — than participants without asthma — 16 ppb — ($p<0.001$). We found with further stratification for current smoking status, a higher mean F_{ENO} in non-smokers than in smokers ($p<0.01$), independent of asthma status. An additional stratification resulted in a higher mean F_{ENO} in individuals with concomitant atopy and asthma than in non-asthmatics ($p<0.001$) with atopy. Similarly, non-atopic individuals with asthma had a higher mean F_{ENO} compared to non-atopic non-asthmatic individuals ($p=0.016$). See Table 4.7 for stratified results.

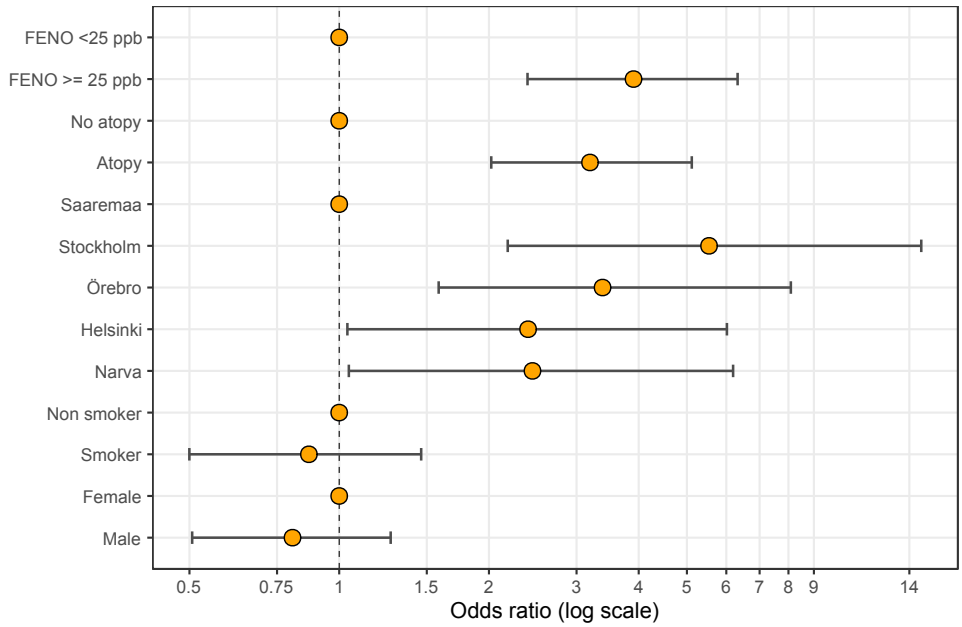


Figure 4.8: Binary logistic regression model with asthma as outcome and F_{ENO} ≥25 ppb, participating centre, gender, smoking, and atopy as predictors. Presented as odds ratio (OR) with 95% confidence intervals. Reference groups: F_{ENO}<25 ppb, no atopy, Saaremaa, non-smoker and female. *From:* *Lassmann-Klee et al. (2020).*

Table 4.7: Fractional exhaled nitric oxide (F_{ENO}) mean (SD) stratified by smoking and atopic status in participants with or without asthma. *From* *Lassmann-Klee et al. (2020)*

		Asthmatics (n=103)				Non-asthmatics (n=1329)			
F_{ENO} (ppb)	mean (sd)	27 (29)				16.3 (11)			
		current non-smoker		current smoker		current non-smoker		current smoker	
	n (%)	80 (73%)		23 (22%)		903 (68%)		422 (32%)	
F_{ENO} (ppb)	mean (sd)	29.8 (31.6)		17.1 (12.9)		18 (11.6)		12.8 (8.8)	
F_{ENO} ≥25 ppb	n (%)	32 (40%)		4 (17%)		142 (16%)		26 (6%)	
		atopic	non-atopic	atopic	non-atopic	atopic	non-atopic	atopic	non-atopic
		Σn=67		Σn=21		Σn=841		Σn=393	
	n (%)	42 (63%)	25 (37%)	13 (62%)	8 (38%)	271 (32%)	570 (68%)	130 (33%)	263 (67%)
F_{ENO} (ppb)	mean (sd)	31.5 (37.3)	30.9 (22.5)	21.7 (14.9)	11.3(7.1)	19.9 (14.2)	17 (9.8)	13.3 (11.4)	12.4(7.4)
F_{ENO} ≥25 ppb	n (%)	17 (41%)	13 (52%)	3 (10%)	1 (13%)	53 (20%)	80 (14%)	10 (7.7%)	15 (6%)

Data shown as n (%) or mean (standard deviation).

4.3.5 F_{ENO} ≥25 ppb

When calculating odds ratios for the likelihood of having a F_{ENO} ≥25 ppb, we found that individuals with self-reported asthma, physician-diagnosed asthma, atopy or allergic rhinitis were more likely to have a result ≥25 ppb (Table 4.8). This was also true for individuals with asthma symptoms, nightly symptoms, ICS use, and SABA use — all of which were within the previous year. Current smokers were more likely to have a F_{ENO} result <25 ppb. We found no association between F_{ENO} ≥25 ppb and COPD diagnosis or childhood asthma.

Table 4.8: Univariate analysis (crude odds ratio 95% CIs) for F_{ENO} ≥25 ppb and diagnosis, current smoking, and — within the last year — respiratory symptoms and medication. Total sample (n=1498). *From Lassmann-Klee et al. (2020)*

		Odds ratio	Confidence Intervals	p
Ever asthma	yes	3.71	2.40-5.74	<0.001
Asthma by MD *	yes	2.78	1.74-4.44	<0.001
Asthma in childhood	yes	1.35	0.78-2.33	0.358
Asthma symptoms	yes	3.1	2.06-4.68	<0.001
Nightly symptoms	yes	2.04	1.15-3.60	0.02
Asthma medication	yes	3.22	2.05-5.05	<0.001
ICS † used	yes	2.48	1.34-4.6	0.005
Saba ‡ used	yes	3.40	2.04-5.65	<0.001
Atopy§	positive	1.62	1.20-2.19	0.002
Allergic rhinitis	yes	2.11	1.58-2.83	<0.001
COPD ¶ by MD*	yes	1.09	0.61-1.92	0.897
Current smoking	yes	0.33	0.23-0.49	<0.001

*medical doctor, *inhaled corticosteroids, ‡short-acting β_2-agonist, §skin prick tests, ¶chronic obstructive pulmonary disease

Prevalence of F_{ENO} ≥25 ppb in Nordic populations

In the whole sample, the prevalence of F_{ENO} ≥25 ppb was 14.6%. The prevalence in the Northern European countries was 14.7% in Sweden, 21.4% in Finland, and 11.2% in Estonia. The individual centres had a prevalence of 10.6% in Stockholm, 16.6% in Örebro, 21.4% in Helsinki, 11.4% in Narva, and 11.1% in Saaremaa.

When comparing the likelihood ORs (95% CIs) of a F_{ENO} result ≥25 ppb between the populations in the Northern European countries with a binary logistic regression model, we found that compared to Finland, the ORs were 0.60 (0.41–0.89) in Sweden and 0.48 (0.33–0.72) in Estonia, and adjusted for positive SPT 1.52 (1.11–2.07), smoking 0.33 (0.22–0.49), and male gender 1.36 (1.00–1.85). A similar comparison for F_{ENO} ≥25 ppb within the study centres resulted in adjusted ORs of 0.42 (0.21–0.81) in Stockholm, 0.65 (0.43–0.98) in Örebro, 0.53 (0.32–0.84) in Narva and 0.45 (0.28–0.71) in Saaremaa, compared with Helsinki.

Schreiben ist gut, Denken ist besser.

Klugheit ist gut, Geduld ist besser.

Writing is good, thinking is better.

Cleverness is good, patience is better.

Hermann Hesse, Siddhartha

5

Discussion

This chapter offers a critical analysis of our findings, firstly exploring the research methodology, secondly synthesising our main findings and providing a detailed thematic examination, and thirdly evaluating the importance of our study.

5.1 Methodological considerations

F_{ENO} mouthwashes: considerations

This dissertation is partially focused on the methodology of F_{ENO} acquisition. There are multiple factors linked to F_{ENO} measuring which can produce a measurement error within the scope of systematic errors, apart from errors attributable to randomness. The findings of this dissertation offer a mouthwash method to reduce systematic bias in F_{ENO} measurement, through minimising oral NO contamination. This systematic bias may be of concern in respiratory research, but the error may also be linked to inter-individual variation, and its minimisation could arguably reduce random bias.

F_{ENO} acquisition: considerations

Further sources of measurement error derive from the properties of the NO analysers and how exact the F_{ENO} results they produce are. Within this scope falls the precise calibration of the devices. External NO sources, like ambient air, can affect the F_{ENO} results, nevertheless modern devices contain filters preventing NO ambient contamination. Other NO sources are dietary, and are reduced through strict measuring protocols. During individual F_{ENO} acquisition, bias is minimised through obtaining a mean value from at least two single determinations, therefore ensuring its repeatability. The F_{ENO} values are obtained from the end of expiration, where a plateau F_{ENO} value is constant. The F_{ENO} value and expiratory flow are held constant by applying counter-pressure resistors. Another factor to be considered is the variation which can be observed through repeated measurements, i.e., intersession repeatability. Previously, Ekroos et al. (2002) found good reproducibility of F_{ENO} in healthy participants and asthmatics.

F_{ENO} multiple flow and validation: considerations

Another error origin while measuring F_{ENO} is the inverse proportionality of F_{ENO} and expiratory flow rate, i.e., the higher the flow rate, the lower the F_{ENO} and vice versa. Not accounting for an exact standardised flow rate delivers values without repeatability and comparability. Through our developed model, F_{ENO} can be standardised and compared independently of the original expiratory flow rate. The model was validated in several populations with different characteristics (see Table 3.1), and proved robust in epidemiological research.

Epidemiological methods: considerations

The population was selected through a random procedure providing a representative selection of the general population, nevertheless the procedure can produce a selection bias. This bias can be directly related to the participation rate, with groups of individuals with certain intrinsic characteristics deciding to participate in the study or ignore the invitation. The original postal questionnaire in this study had good participation rates — 72–89% — depending on the study centre. The time elapsed for starting the clinical interview varied depending on the centre, from 1 to 4 years. The response rate declined during the clinical interview — 37–67% — probably due to the nature of being a follow-up study. The invitations for the clinical interview were sent to a random selection of those who returned the postal questionnaire, aiming to minimise the bias. It still remains open if the more afflicted individuals were more prone to participate in the clinical interview and whether this varied throughout the centres. Nevertheless, previous epidemiological studies in Sweden showed that non-responders had a similar prevalence of respiratory diseases and symptoms as responders (Rönmark et al., 2009). A previous study by Rönmark et al. (1999) found that among non-responders the prevalence of men, smoking, and manual workers was higher, as well as the prevalence of respiratory diseases and symptoms. Hypothetical differences between responders and non-responders may differ depending on the characteristics of the population, like age, nationality, ethnicity, lifestyle, education, morbidity,

and these differences may directly affect the response rate.

We applied the same questionnaire (in regional languages) to all studied populations. The main questions during the interview were identical with the postal questionnaire, but the clinical interview contained additional questions. This approach guaranteed a continuity in the results from the previous study, but measuring F_{ENO} simultaneously with the clinical interview further supported the results.

5.2 Discussion of main results

5.2.1 Mouthwashes and F_{ENO}

Although mouthwashes are popularly used in everyday life, their application in clinical F_{ENO} acquisition is still not widely implemented, and the mechanisms of action are not thoroughly elucidated, but probably act by reducing oral NO contamination (American Thoracic Society and European Respiratory Society, 2005). Nevertheless, the use of mouthwashes in physiological research of F_{ENO} is endorsed by current guidelines (Horváth et al., 2017). Here we applied common mouthwashes — tap and carbonated water — to investigate their effect on F_{ENO} at the flow of 50 mL/s. We demonstrate that tap water — with an alkaline pH of ca. 8 — lowered F_{ENO}, but had a brief effect. Zetterquist et al. (1999) observed a similar short effect on F_{ENO} with an alkaline — pH 7.85 — $NaHCO_3$ solution.

In contrast, the carbonated water mouthwash had a prolonged effect of lowering F_{ENO}, compared to tap water. The tap water mouthwash lowered the oral F_{ENO} only for 2 minutes. This might result from the mildly acidic pH — 5.4–5.5 — of carbonated water, which resembles the acidity of saliva — pH of 6–7 (Humphrey and Williamson, 2001). The chemical properties of carbonated water probably inhibit oral NO, as opposed to a stimulation of NO production by highly acidic solutions (Zetterquist et al., 1999).

The role of the mouthwashes' pH is further illustrated by previous investigations with neutral pH, a phosphate buffer saline solution (Gaston et al., 2006) and distilled water (Zetterquist

et al., 1999), without evident changes in F_{ENO} levels.

5.2.2 Mouthwashes and F_{ENO}: multiple flows

We further showed that the mouthwash procedure can effectively reduce F_{ENO} acquired at a range of expiratory flows from 30 mL/s to 300 mL/s, with a clear augmented lowering effect of carbonated water compared to tap water. As mentioned above, the mouthwashes can affect the measured F_{ENO} by allegedly interfering with the oral NO production. To prove this, we modelled extended exhaled NO parameters, which can distinguish between the maximum airway NO flux ($\dot{J}_{awNO}$) and the alveolar NO concentration (C_{ANO}) and provide values for the NO airway diffusion (D_{awNO}). Our results showed that, when comparing the tap water and carbonated mouthwashes, the carbonated mouthwash only reduced NO from the airways, i.e., lowering $\dot{J}_{awNO}$ without differences in the alveolar NO concentration or airway NO diffusion. Previous researchers found similar results in relation to $\dot{J}_{awNO}$ and C_{ANO}, when applying a chlorhexidine mouthwash (Heijkenskjöld-Rentzhog et al., 2012).

5.2.3 Antibacterial properties of mouthwashes in relation to F_{ENO}

The chemically active solutes in mouthwashes, such as tap or carbonated water, have antimicrobial properties, which could partly explain the effect of the mouthwashes in reducing oral NO. Tap water usually contains small amounts of added chlorine (for example, in Helsinki) to ensure a delivery of tap water without pathogenic bacteria. During the mouthwash procedure, the chlorine could act as an antiseptic. The solutes in carbonated mineral water used in the present study — different salts — could also act anti-microbially, for example $MgCl_2$ and $CaCl_2$ inhibit bacterial growth in an acidic environment (Alarcón et al., 2014), and the salts in tap water could have an antiseptic effect. Nevertheless, the pH of the mouthwash solution plays an important role in F_{ENO} reduction, not only of the solution itself, but probably the pH of the oral surface mucosa as well. A well-known antibacterial component of commercially avail-

able mouthwashes is chlorhexidine, which has a proven long-lasting effect (Zetterquist et al., 1999) on reducing oral NO (Heijkenskjöld-Rentzhog et al., 2012). However, development of bacterial resistance to chlorhexidine should be avoided in routine or large-scale testing (Horner et al., 2012). The effect of chlorhexidine on F_{ENO} is probably due to its antiseptic cause than from its alkaline pH (pH 8).

5.2.4 Novel F_{ENO} conversion model

F_{ENO} values are flow dependent, therefore the respiratory research community previously adopted a standard flow value of 50 mL/s to clinically assess eosinophilic airway inflammation (Dweik et al., 2011). Despite this fact, values acquired at different expiratory flows need a conversion for comparison in epidemiological research. We provided here a novel non-linear method with a simple application of an equation for providing estimates of $\hat{F}_{ENO}$ for different flows. We tested the model in several external populations and observed a high validity of the estimates at the population level. Nevertheless the method may provide erroneous results at the individual level since it employs a general fixed estimate of the alveolar concentration C_{ANO} as the slope and calculates the estimated $\hat{F}_{ENO}$ at a given flow from the intercept ($\dot{J}_{\text{awNO}}$), which is modelled from the acquired F_{ENO} and flow. The method best suits mixed populations, since among only asthmatics or alveolitis patients it performs with some degree of error. To address this issue, adjustments to the model would be needed, either to the applied $\dot{J}_{\text{awNO}}$ or C_{ANO}. An alternative application of our non-linear model is calculating extended NO parameters — C_{ANO} and $\dot{J}_{\text{awNO}}$ — from a multiple-flow NO dataset, but this falls outside the aims of this dissertation.

After validation, we applied our model in epidemiological asthma research, as described previously and discussed below.

5.2.5 F_{ENO} and asthma epidemiology in Northern European countries

West–east gradient of allergy and F_{ENO}

The most remarkable finding of the present study was the west–east gradient in the included population from Sweden, Estonia, and Finland, of asthma prevalence, asthma symptoms, and F_{ENO} level. The population in Estonia had lower F_{ENO} values, and a lower likelihood of having asthma. The exact causes for the disparity in this population are unknown, due to the design of the study — cross-sectional — we can only hypothesise its causality. A multifactorial model can help in explaining this disparity of asthma in different populations. There are different factors attributable to the differences in asthma prevalence and risk, and the west–east gradient of F_{ENO}. At the individual level: genetic predisposition, epigenetics, behaviour, and co-morbidities. At the interpersonal level: exposure in the household to siblings, pets, and smoking. At the environmental level: pollution, air quality, and allergens. There are also factors which increase the burden of asthma, its awareness, and morbidity: access to healthcare and healthcare quality, medical practice, diagnostic procedures and medication. From the last mentioned, medication obviously influences morbidity through patient compliance, costs, reimbursement and therefore also through health policies, which are societal and community levels. This illustrates how the factors are also interrelated and exhibit different levels of complexity.

Allergic sensitisation

When comparing the populations in Sweden, Finland and Estonia, previous studies found differences in allergic sensitisation; pollen and furry animals were main allergens in Sweden and Finland (Pallasaho et al., 2006; Warm et al., 2013), while storage mites and cockroaches dominated in Estonia (Raukas-Kivioja et al., 2007). Similar differences in sensitisation were found in North Karelia in Finland, compared with the Republic of Karelia in Russia (Vartiainen et al.,

2002).

Protective factors

Living on a farm has previously been found to lower the risk of allergy and asthma (Borna et al., 2019; Lambrecht and Hammad, 2017; Wennergren et al., 2010). In the present study, the studied centre in Saaremaa, Estonia, with the lowest asthma prevalence and likelihood of asthma, and a low F_{ENO} median, is an island with a rural environment, therefore its population could be protected by farm living.

Pollution

The air concentrations of benzene, phenol, and fine particles in the highly industrial area of Narva in Estonia could add to explain the lowest F_{ENO} median, despite a higher prevalence and likelihood of asthma than in Saaremaaa. Recently, Orru et al. (2018) found a similar asthma prevalence in Narva as in our study, when analysing the high pollution in eastern Estonia.

Smoking

We observed here, as expected, lower F_{ENO} levels in current smokers. Several preceding investigations reported a similar effect of smoking on F_{ENO} (Juusela et al., 2013; Kostikas et al., 2008; Taylor et al., 2007). Other studies also reported a decrease of F_{ENO} levels in former smokers (Juusela et al., 2013; McSharry et al., 2005). The effect of smoking could be explained by a down-regulation of an inducible nitric oxide synthases (iNOS), responsible for the F_{ENO} production in the lung epithelial cells (Hoyt et al., 2003). These previous studies corroborate our findings regarding the strong effect of smoking on decreasing F_{ENO}, since our stratified analyses show that independently of asthma or atopy, current smokers exhibited lower F_{ENO} than non-smokers.

Atopy

Through our findings, we confirmed previous research on the association of atopy and F_{ENO} levels in Scandinavian populations (Çolak et al., 2018; Olin et al., 2006; Thorhallsdottir et al., 2016), and showed that this association also prevails in general populations stemming from multiple centres across Northern European countries. Here we showed that atopy increases the likelihood of asthma. Nevertheless, in healthy asymptomatic non-smokers, F_{ENO} values are similar regardless of atopic status, according to a previous study in Helsinki (Rouhos et al., 2008). This indicates that in atopic individuals, a high F_{ENO} value signals an allergic airway disease. As shown in the present study, atopic individuals usually exhibit a high F_{ENO}, this irrespective of smoking, and corroborates large Scandinavian study (Olin et al., 2006).

5.3 Importance of results

Mouthwashes

Our studies show that a carbonated water mouthwash can reduce oral NO for a time span of 12 min and is suitable for routine F_{ENO} testing, possessing ideal attributes: neutral taste and commercially affordable. Applying a mouthwash could affect clinical decisions when acquiring F_{ENO} and provide more accurate values. Additionally, its use could be endorsed in F_{ENO} research procedures.

Extended F_{ENO} modelling

The novel extended F_{ENO} model presented here can provide estimated F_{ENO} values for different flows when a single F_{ENO} determination is available, and it could be used, apart from converting F_{ENO} values, to provide a control F_{ENO} value (for a standard flow 50 mL/s), when employing an extended NO model such as the Högman-Meriläinen algorithm (Högman and Lehtimäki, 2020). An important future application of our novel model is providing extended

NO parameters, the maximum airway NO flux $\dot{J}_{awNO}$ and the alveolar NO concentration C_{ANO}, but its implementation is the subject of future research.

Epidemiology of asthma in Northern European countries

A valuable contribution of our study is showing the parallel differences between F_{ENO} levels and asthma, asthma symptoms and allergy, and how these parallels can be interpreted as a gradient when comparing different countries or areas. Therefore, it corroborates the west–east disparity of allergic diseases.

Analysis of F_{ENO} in Northern European Countries

Our study provides an insight into the epidemiology of F_{ENO} and asthma in general populations of selected Northern European countries (Sweden, Finland, and Estonia), and although there are larger single centre F_{ENO} studies in Northern European countries, our study represents the largest analysis of F_{ENO} in a multicentric Nordic population and shows the differences between the countries.

Nadie puede durar tanto, no existe ningún recuerdo por intenso que sea que no se apague.
No one can last forever, there is no memory, no matter how intense, that does not extinguish.

Juan Rulfo, El llano en llamas

6

Conclusions

A mouthwash with carbonated mineral water lowers F_{ENO} for a time of 12 min and is therefore suitable for reducing oral contamination in physiological research and is also applicable in clinical routine asthma assessment.

In extended F_{ENO} analysis, the mouthwash with carbonated mineral water reduces oropharyngeal contamination, expressed as a reduction of the maximum airway NO flux ($\dot{J}_{awNO}$), without affecting the capacity of the airways of NO diffusion (D_{awNO}) or the alveolar NO concentration (C_{ANO}).

A conversion of F_{ENO} values acquired at different flow levels was achieved by applying a

novel non-linear model, with its main asset of converting F_{ENO} values to a standard flow of 50 mL/s. The model was validated in healthy and mixed populations, as well as with obstructive pulmonary disease patients. Another advantage of the model is the ability to provide extended F_{ENO} values ($\dot{J}_{awNO}$ and C_{ANO}), and should be the subject of further research.

The model was applied in general populations samples from Sweden, Finland, and Estonia by converting F_{ENO} values to a standard flow. The populations of Sweden and Finland showed a higher prevalence of asthma and allergic airway inflammation, compared to Estonia. Within these pooled populations, F_{ENO} and atopy were associated with a higher likelihood of asthmatic disease. The epidemiological findings corroborate the west-east disparity of allergic diseases with quantifiable clinical methods (F_{ENO} and skin prick tests).

Acknowledgments

My most sincere gratitude goes to both my supervisors, Prof. Päivi Piirilä and Prof. Anssi Sovijärvi, for introducing me to the exciting world of pulmonary physiology and research, for providing me with accurate guidance through my doctoral journey and for their inexhaustible support. And also for their scientific, critical minds, always attentive to improvements or errors.

This dissertation was feasible through the seed planted by Prof. Bo Lundbäck and Prof. Anssi Sovijärvi that grew into a research network between Nordic Countries and Estonia, and allowed this work through originally designing the FinEsS research project in the 1990s. The FinEsS evolved in 2017 into the current Nordic EpiLung project, a research network between Sweden, Norway and Finland established by Prof. Eva Rönmark, which played an important position as funder of this thesis, and is specially thanked.

I want to offer a word of thanks to the University of Helsinki, the Faculty of Medicine, the Doctoral School of Health, and the Doctoral Programme in Clinical Research, for the teaching and financing provided.

There are no words that can express the debt of gratitude to all the supporters of this dissertation: the Nummela Sanatorium Foundation; Foundation of the Finnish Anti-Tuberculosis Association; Finland; Research Foundation of the Pulmonary Diseases, Finland; Tampere Tuberculosis Foundation: Eero Hämäläinen, Finland; Väinö and Laina Kivi Foundation, Finland; Ida Montin Foundation, Finland; University of Helsinki, Finland; Helsinki University Hospital, Finland; and the Nordic Council of Ministers NordForsk (Nordic EpiLung Study), Norway.

I appreciate the researchers in the Nordic EpiLung network, PhD Helena Backman, PhD Ben Brumpton, Prof. Hannu Kankaanranta, Prof. Arnulf Langhammer, Prof. Lauri Lehtimäki, and Prof. Eva Rönmark, who apart from co-authoring the original publications included in this thesis, provided useful comments and shared experiences during meetings. I am grateful for the scientific input provided by the further co-authors of the publications: Prof. Lauri Halonen, PhD Maria Juusela, PhD Matz Larsson, MD Tuula Lindholm, Ass.Prof. Ari Lindqvist, Ass.Prof. L. Pekka Malmberg, PhD Mari Meren, Ass.Prof. Markus Metsälä, PhD Jaak Põlluste, PhD Annamari Rouhos, and PhD Britt-Marie Sundblad.

My thesis committee members, Ass.Prof. Paula Kauppi and Ass.Prof. Antti Loimaala, deserve sincere thanks through providing guidance at our yearly meetings. I deeply appreciate the opportunity to work at the Laboratory of Clinical Physiology and Nuclear Medicine provided

by Ass.Prof. Petri Haapalahti, PhD Lauri Karhumäki, and Ass.Prof. Antti Loimaala, and for facilitating work spaces in the laboratory.

The reviewers of this dissertation, Ass.Prof. Hanna Mussalo and Prof. Tuula Vasankari, are acknowledged for their valuable comments and assessment. My sincere gratitude goes to PhD Alyce Whipp for the fast and accurate language editing of this dissertation and publications.

All previous researchers and collaborators who in any form assisted the FinEsS research project deserve my gratitude, in particular PhD Annette Kainu and PhD Paula Pallasaho. Research nurse Kerstin Ahlskog is acknowledged for managing the research data.

I warmly thank the staff members from the Skin and Allergy Hospital, the Finnish Institute of Occupational Health Helsinki and the Laboratory of Clinical Physiology at the Meilahti Hospital for providing aid during the data acquisition.

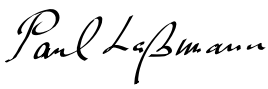
The University of Helsinki PhD Students Association (HYVÄT) for their activities and advocacy.

My parents, Claudia and Guenther, for their infinite support during my studies, and my siblings, Erich and Katherine, for their encouragement.

My friends, Filo, Ilari, and Rene, for their amusing shared occasions and philosophical discussions.

A very special feeling of thankfulness pervades for my family, Elina, Paulina, Amalia and Sampo, for providing me with infallible love, understanding and joy.

Fiskars, October 2021



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Scandinavian Journal of Clinical and Laboratory Investigation

ISSN: 0036-5513 (Print) 1502-7686 (Online) Journal homepage: http://www.tandfonline.com/loi/iclb20

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To cite this article: Paul Guenther Lassmann-Klee, Tuula Lindholm, Markus Metsälä, Lauri Halonen, Anssi Raimo Antero Sovijärvi & Päivi Piirilä (2018) Reduction of F_{ENO} by tap water and carbonated water mouthwashes: magnitude and time course, Scandinavian Journal of Clinical and Laboratory Investigation, 78:1-2, 153-156, DOI: 10.1080/00365513.2017.1419574

To link to this article: https://doi.org/10.1080/00365513.2017.1419574

SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION, 2018
VOL. 78, NO. 1-2, 153–156
https://doi.org/10.1080/00365513.2017.1419574

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TECHNICAL NOTE

Reduction of F_{ENO} by tap water and carbonated water mouthwashes: magnitude and time course

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ABSTRACT

Fractional exhaled nitric oxide (F_{ENO}) assesses eosinophilic inflammation of the airways, but F_{ENO} values are also influenced by oral nitric oxide (NO). The aim of this pilot study was to measure F_{ENO} and compare the effect of two different mouthwashes on F_{ENO} and analyse the duration of the effect. F_{ENO} was measured in 12 randomized volunteers (healthy or asthmatic subjects) with a NIOX VERO® analyser at an expiratory flow rate of 50 mL/s. After a baseline measurement, a mouthwash was performed either with tap water or carbonated water and was measured during 20 min in 2 min intervals. The procedure was repeated with the other mouthwash. We found that both mouthwashes reduced F_{ENO} immediately at the beginning compared to the baseline ($p < .001$). The carbonated water mouthwash effect lasted 12 min (p ranging from <0.001 to <0.05). The tap water mouthwash reduced F_{ENO} statistically significantly only for 2 min compared with the baseline. We conclude that a single carbonated water mouthwash can significantly reduce the oropharyngeal NO contribution during a 12 min time interval.

ARTICLE HISTORY
Received 2 June 2017
Revised 4 December 2017
Accepted 17 December 2017

KEYWORDS
Fractional exhaled nitric oxide (F_{ENO}); carbonated water; mouthwash; tap water; asthma; inflammation; laboratory standardization

Introduction

The fractional concentration of exhaled nitric oxide (F_{ENO}) rises during eosinophilic airway inflammation and its widespread application as biomarker facilitates asthma diagnosis [1,2]. Although oral nitric oxide (NO) production contributes to F_{ENO} values, a routine mouthwash remains widely unimplemented. The ATS/ERS guidelines (2005) suggest that a mouthwash may reduce oral contamination and the European Respiratory Society's Task Force (2017) recommends a mouthwash only in physiological investigations [3,4]. However, the influence of mouthwashes continues partially unaddressed. Piirilä et al. [5] demonstrated the reduction of F_{ENO} after a carbonated water mouthwash (pH 5.4–5.5). Analogously, Heijkensköld-Rentzhog et al. [6] and Zetterquist et al. [7] showed that an antiseptic chlorhexidine mouthwash (pH 8) caused a significant and long lasting decrease in F_{ENO}. According to Zetterquist et al., mouthwashes differ in influencing F_{ENO} in magnitude and time: F_{ENO} diminished minimally after a distilled water mouthwash (pH 7), on the other hand, a 10% sodium bicarbonate solution mouthwash (pH 7.85) reduced F_{ENO} significantly. This contrasted to a 3% ascorbic acid solution (pH 2.5) mouthwash, which stimulated F_{ENO} production [7]. Gaston et al. proposed that F_{ENO} levels underlie changes in airway pH and demonstrated that a neutral buffer mouthwash (pH 7) has no effect on F_{ENO} levels [8].

In Finland, a mouthwash is routinely used prior to measuring of F_{ENO}, either with tap water or with carbonated water. Particularly, in our laboratory in Helsinki, carbonated water is employed. Nevertheless, no previous studies have elucidated how the effect of tap water and carbonated water mouthwashes on F_{ENO} values differs in magnitude and duration. The aim of this pilot study was to investigate the effect of a carbonated water mouthwash on F_{ENO} as a function of time starting from a baseline and to compare the effect to a tap water mouthwash.

Methods

We recruited 12 healthy volunteers, non-smoking healthcare workers, aged 27–63 years. Three have previously had diagnosis of asthma and used inhaled corticosteroids regularly. Participants were included without further selection. The volunteers had mean (SD) height of 179 (9) cm and weight of 82 (21) kg. F_{ENO} was measured during one sitting and the expiratory flow rate used was 50 mL/s. We used a NIOX VERO® analyser according to the instructions from the manufacturer [9]. The recommendations according ATS/ERS were followed [3]. The subjects refrained from drinking coffee 2 h, and from eating and drinking 1 h before the study. Strenuous exercising prior measurement was discouraged.

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Supplemental data for this article can be accessed here.

A testing array consisted of a baseline measurement (prior to mouthwash), followed by a mouthwash with a duration of approximately 30 s and an immediate F_{ENO} measurement (time zero). A baseline measurement before each mouthwash was acquired from 2 to 4 determinations obtaining a mean. If the difference of F_{ENO} values for the baseline was >1 ppb, additional exhalations were performed. The testing array continued with single determinations at intervals of 2 min until 20 min. The test was repeated with the other mouthwash about 15 min later.

The order of the mouthwashes was carried out in a manner that the first participant started with a tap water mouthwash followed by a carbonated water mouthwash. The following participant had an inverted order of the mouthwashes. The order for the next volunteer was again reverted. We continued in that fashion until all volunteers were recruited and tested.

The mouthwash consisted of rinsing the oral cavity for approximately 30 s with 100 mL either of tap water or carbonated water. The tap water used had a pH of 8.3 and the following solute concentrations: Cl^- 5 mg/L, Ca^{2+} 22 mg/L, Na^+ 6 mg/L, K^+ 1.4 mg/L, SO_4^{2-} 10 mg/L, and ClO_2 0.5 mg/L [7]. The carbonated water used for the mouthwash was a bottled drink and had an estimated pH of 5.7–5.9 and contained $NaHCO_3$, $KHCO_3$, $MgCl_2$, and $CaCl_2$ (HARTWALL VICHY ORIGINAL®, Oy Hartwall Ab, Helsinki, Finland) (Personal communication with Riitta Saleva-Sjöblom from Hartwall Ab; unreferenced). Concentration values were unavailable. We followed the ethical principles stated in the declaration of Helsinki. In addition, each participant gave a written consent and the study was approved by the ethics committee of the Helsinki University Central Hospital (HUS/1417/2016 task 13.2.01).

Statistics

Analysis was performed using IBM® SPSS® statistics software version 22 (IBM Corporation, Armonk, NY) and GRAPHPAD® PRISM® version 5.04 (Graphpad Software, Inc., San Diego, CA). We accepted a significance level of $\alpha = 0.05$ as statistically significant. We tested the variables with a Shapiro–Wilk test, which confirmed that they were normally distributed. Differences in the F_{ENO} values between mouthwash procedures in time were tested with a general linear model (GLM) for repeated measures. F_{ENO} is presented as an estimated marginal mean in ppb, 95% confidence interval (CI) [lower bound; upper bound]. The graphical material was obtained with the statistical software GRAPHPAD® PRISM® version 5.04 and graphically presented as an arithmetic mean (ppb), 95% (CI).

Results

F_{ENO} declined significantly immediately after the tap water mouthwash from the F_{ENO} baseline of 18.1 ppb (estimated marginal mean), 95% CI [13.1; 23.2] to 15.7 ppb, 95% CI [10.7; 20.7] ($p < .001$). After 2 min, F_{ENO} (tap water) increased to 17 ppb, 95% CI [12.1; 21.9]. A significant difference was found compared to the baseline ($p = .004$). During the consecutive measurements (4–20 min) there were no significant differences of F_{ENO} (tap water) compared to the baseline, apart from the measurement at 14 min, where F_{ENO} was higher: 19.2 ppb, 95% CI [14.0; 24.4].

After the carbonated water mouthwash, F_{ENO} declined immediately and significantly to 14.6 ppb, 95% CI [10.1; 19.2] ($p < .001$) compared with the F_{ENO} baseline of 17.9 ppb (estimated marginal mean), 95% CI [12.9; 22.9]. F_{ENO} (carbonated water) stayed significantly lower ($p < .05$) compared with the baseline during the interval of 2–12 min. At 14 min, F_{ENO} (carbonated water) increased to 17.6 ppb, 95% CI [12.7; 22.6] and there was neither a statistical difference at that point nor during the consecutive measurements. Individual results are visualized in Figure 1 including the first two minutes. Individual data for the first ten minutes and baseline are included as a Supplemental file.

When comparing the differences between mouthwashes in relation to time and the baseline (pairwise comparisons), the estimated marginal mean of F_{ENO} was significantly lower ($p = .008$) after the carbonated water mouthwash (F_{ENO}: 17.0 ppb, 95% CI [12.0; 22.1]) than after the tap water mouthwash (F_{ENO}: 18.0 ppb, 95% CI [12.9; 23.1]). When comparing the F_{ENO} differences between mouthwashes (pairwise comparisons), there was no significant difference immediately after the mouthwash, i.e. at time zero ($p = .083$). At the 2 min measurement point, F_{ENO} (carbonated water) was significantly lower than F_{ENO} (tap water) ($p = .03$). Differences were also significant at the next time points: 4 min ($p = .015$), 8 min ($p = .037$), 12 min ($p = .005$), and 14 min ($p = .021$). Differences were not significant at 6 min ($p = .141$), 10 min ($p = .056$), 16 min ($p = .736$), 18 min ($p = .196$), and 20 min ($p = .232$). These main results as arithmetic means (95% CI) are visualized in Figure 2.

Discussion

We found that the overall effect of the carbonated water mouthwash in lowering of F_{ENO} was significantly larger than the effect of the tap water mouthwash ($p = .008$). Immediately after the mouthwashes, both mouthwashes lowered F_{ENO} on a highly significant level ($p < .001$) compared with the baseline, but the effect of tap water decayed rapidly. The statistically significant effect of the tap water mouthwash vanished after 2 min. The significant effect of the carbonated water mouthwash in lowering F_{ENO} endured for 12 min.

When making pairwise comparisons, F_{ENO} after the carbonated water mouthwash was lower than after the tap water mouthwash from time zero until 14 min, but the difference was not always significant. This might be due to the small number of subjects.

Here we demonstrate that an alkaline tap water mouthwash has a significant, but a short-lasting effect of lowering F_{ENO} levels. This could be due to the alkaline pH and the low concentrations of chemically active solutes in the tap water provided by the communal water service. In Helsinki, tap water quality is regulated by law and is required to have a moderately alkaline pH value (pH >8). Chemically active

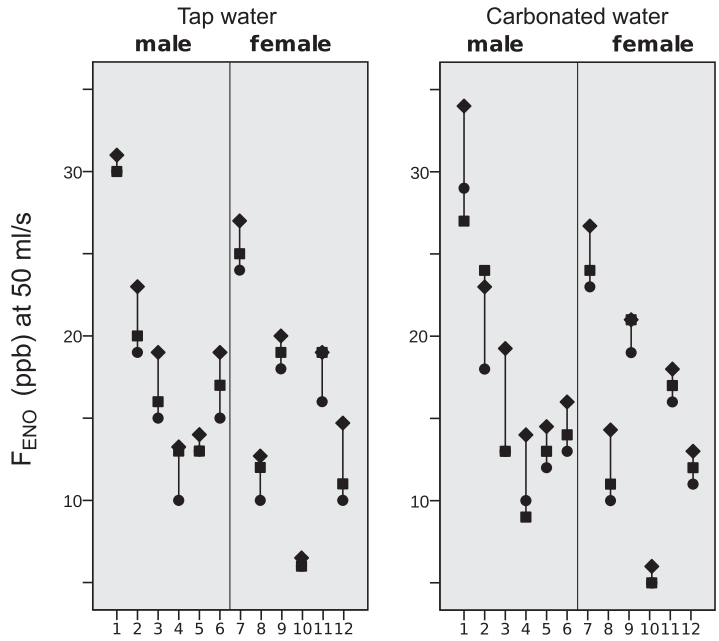

Figure 1. F_{ENO} (ppb) after tap water mouthwash (left side) and carbonated water mouthwash (right side). Individual F_{ENO} values ($n = 12$) separated by gender, during baseline (rhombi), immediately after mouthwash (squares), and after 2 min (circles).

solutes may have a low antiseptic effect, e.g. magnesium chloride and chlorine. (Personal communication with Kirsi-Marja Hiillos from Helsinki Region Environmental Services Authority HSY; unreferenced) [10]. These overall characteristics might explain the short acting effect of tap water on oral F_{ENO}. We suggest that the present study is the first one to investigate the effect of a tap water mouthwash on F_{ENO} values. Zetterquist et al. reported previously that a 10% $NaHCO_3$ (pH 7.85) solution reduced F_{ENO} release for only 1 min [7]. In the present study, the effect of the tap water mouthwash on the F_{ENO} levels resembles that reported by Zetterquist using $NaHCO_3$. This could possibly be explained by the alkaline pH of both solutions.

To further clarify the influence of the mouthwashes' pH on F_{ENO} values, we may mention a previous investigation with a pH neutral phosphate buffer saline solution performed by Gaston et al. [8]. The neutral pH mouthwash showed no evident decrease in F_{ENO}. Similarly, Zetterquist et al. [7] found that a mouthwash with distilled water (neutral pH) gave a small decrease in F_{ENO}, but without reaching statistical significance.

In comparison with tap water, the carbonated water mouthwash reduced F_{ENO} values for a longer time period. The main chemical difference between the carbonated drink and tap water is the mildly acidic pH of carbonated water (5.4–5.5) which is due to carbonic acid. Additionally, carbonated water contains low levels of $NaHCO_3$, $KHCO_3$, $MgCl_2$, and $CaCl_2$. The pH of the carbonated mouthwash is slightly below the normal physiological pH of saliva, which varies between 6 and 7 [11]. The mildly acidic pH value of

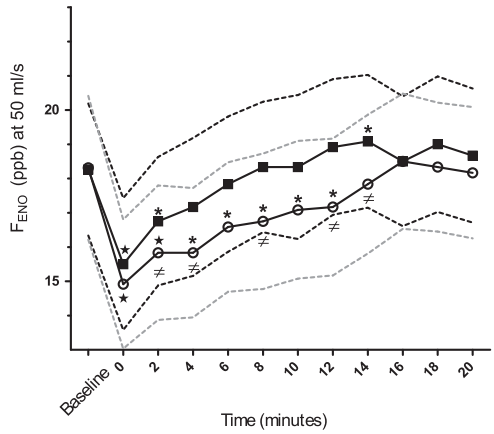

Figure 2. F_{ENO} (ppb) after tap water mouthwash (rectangles) and carbonated water mouthwash (circles) in relation to time (min) and baseline. Baseline F_{ENO} obtained prior mouthwash. Data presented as arithmetic mean. Dotted lines represent the 95% CI (grey dotted line represents the 95% CI for carbonated water and black dotted line represents the 95% CI for tap water). Tested with GLM for repeated measures. Legend: $^*p < .001$ compared with baseline, $^*p < .05$ compared with baseline, $\neq p < .05$ pairwise comparison.

carbonated water seems to inhibit oral F_{ENO} production, in contrast to the stimulation and rise in F_{ENO} values observed after a highly acidic mouthwash [7]. These overall findings reinforce the hypothesis of the pH-dependent influence of mouthwashes on F_{ENO}.

It has been shown before that a fraction of F_{ENO} arises in the oropharynx [12], due to bacterial production of nitrite

[13] and subsequent reduction of nitrite to NO [7,8]. The exact mechanism of how a carbonated mouthwash affects the F_{ENO} levels requires further study, using mouthwashes with distinct chemical composition and different pH values (from acidic to neutral and alkaline). Based on the results of the present study, rinsing the oral cavity with carbonated water effectively reduces the oral F_{ENO} contribution and may, thus, enable a more accurate measurement of F_{ENO} arising from the lower respiratory tract. To determine if the carbonated water or tap water mouthwash procedure affects only the oral contribution to F_{ENO}, without affecting the alveolar concentration of NO or its alveolar diffusion, requires further investigation. Preceding investigations observed an unaffected alveolar concentration of NO through chlorhexidine mouth-washing [6].

Previously, the long-lasting effect on F_{ENO} of chlorhexidine has been shown [7]. A chlorhexidine solution may be efficient in reducing oral NO [6], but it has a long-lasting effect and due to hypothetical development of bacterial resistance may be unsuitable for repeated or large-scale tests [14]. Clorhexidine's pronounced effect on F_{ENO} probably stems from its antibacterial properties and not from the alkaline pH (pH 8). Accordingly, we did not investigate a mouthwash with chlorhexidine.

Although the number of subjects in this study was relatively small, the measurements were carefully performed and clear results were obtained. The equipment employed has an analysis duration of 1 min and 10 s and this imposed the limitation of performing only 1 determination every 2 min. When making pairwise comparisons between mouthwashes, F_{ENO} after the carbonated water mouthwash was lower than after the tap water mouthwash from time zero until 14 min, but the difference was not always significant. This might be due to the small number of subjects.

We conclude that the magnitude and duration of the mouthwash's effect on F_{ENO} levels depends on the properties of the mouthwash's solution, probably on the pH and as well on its antibacterial qualities. Ideally, a mouthwash solution should reduce oral F_{ENO} production effectively, be affordable and easily accessible, and possess a pleasant taste. A carbonated water mouthwash, with a mildly acidic pH resembling that of human saliva, can effectively lower F_{ENO} for a time span of approximately 12 min and suits physiological research procedures. However, these findings might also be important when considering routine clinical testing and analysing F_{ENO} values near the accepted diagnostic cut-off levels, for which applying a mouthwash could affect clinical decisions.

Acknowledgements

Kirsi-Marja Hiillos and Riitta Saleva-Sjöblom for kindly providing information regarding tap water and carbonated water.

Disclosure statement

No conflicts of interest are declared by the authors.

Funding

This work was supported by the Nummela Sanatorium Foundation (PP 2015), (AS 2016), the Finnish State Funding for University-level Health Research (TYH: 2013354), The Research Foundation of the Pulmonary Diseases (PLK 2017), Tampere Tuberculosis Foundation: Eero Hämäläinen (PLK 2017), Ida Montin Foundation (PLK 2017), and Väinö and Laina Kivi Foundation (PLK 2017).

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Scandinavian Journal of Clinical and Laboratory Investigation

ISSN: 0036-5513 (Print) 1502-7686 (Online) Journal homepage: http://www.tandfonline.com/loi/iclb20

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To cite this article: Paul Guenther Lassmann-Klee, Lauri Lehtimäki, Tuula Lindholm, L. Pekka Malmberg, Anssi Raimo Antero Sovijärvi & Päivi Piirilä (2018) Influence of mouthwashes on extended exhaled nitric oxide (F_{ENO}) analysis, Scandinavian Journal of Clinical and Laboratory Investigation, 78:6, 450-455, DOI: 10.1080/00365513.2018.1497802

To link to this article: https://doi.org/10.1080/00365513.2018.1497802

Published online: 22 Oct 2018.

SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION
2018, VOL. 78, NO. 6, 450–455
https://doi.org/10.1080/00365513.2018.1497802

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ORIGINAL ARTICLE

Influence of mouthwashes on extended exhaled nitric oxide (F_{ENO}) analysis

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ABSTRACT

Fractional exhaled nitric oxide (F_{ENO}) is used to assess eosinophilic inflammation of the airways. F_{ENO} values are influenced by the expiratory flow rate and orally produced NO. We measured F_{ENO} at four different expiratory flow levels after two different mouthwashes: tap water and carbonated water. Further, we compared the alveolar NO concentration (C_{ANO}), maximum airway NO flux (J'_{awNO}) and airway NO diffusion (D_{awNO}) after these two mouthwashes. F_{ENO} was measured in 30 volunteers (healthy or asthmatic) with a chemiluminescence NO-analyser at flow rates of 30, 50, 100 and 300 mL/s. A mouthwash was performed before the measurement at every flow rate. The carbonated water mouthwash significantly reduced F_{ENO} compared to the tap water mouthwash at all expiratory flows: 50 mL/s ($p < .001$), 30 mL/s ($p = .001$), 100 mL/s ($p < .001$) and 300 mL/s ($p = .004$). J'_{awNO} was also significantly reduced ($p = .017$), however, there were no significant differences in C_{ANO} and D_{awNO}. In conclusion, a carbonated water mouthwash can significantly reduce oropharyngeal NO compared to a tap water mouthwash at expiratory flows of 30–300 mL/s without affecting the C_{ANO} and D_{awNO}. Therefore, mouthwashes need to be taken into account when comparing F_{ENO} results.

ARTICLE HISTORY
Received 9 March 2018
Revised 12 June 2018
Accepted 17 June 2018

KEYWORDS
Nitric oxide (NO); alveolar NO concentration (C_{ANO}); asthma; fractional exhaled nitric oxide (F_{ENO}); maximum airway NO flux (J'_{awNO}); mouthwashes; carbonated water; inflammation; respiratory system; exhalation; airway obstruction; oropharynx

Introduction

Eosinophilic inflammation of the bronchial epithelium is a chronic process often leading to bronchial hyperreactivity and airway obstruction. During inflammation, fractional exhaled nitric oxide (F_{ENO}) is mainly produced in the airway epithelium, catalysed by inducible NO synthase (NOS2) [1]. In clinical practice, assessing F_{ENO} facilitates asthma diagnosis [2].

F_{ENO} is a combination of nitric oxide (NO) originating in the lung periphery, the bronchioli, the bronchi and the central large airways, and F_{ENO} depends highly on the expiratory flow rates. NO dynamics in the lung periphery determine to a large extent F_{ENO} measured at higher flow rates, while mainly bronchial NO dynamics determine F_{ENO} measured at slower flow rates [3,4]. F_{ENO} measurement has been previously recommended at the expiratory flow rate of 50 mL/s [5,6]. There is evidence that F_{ENO} values over 50 ppb (>35 ppb in children) measured at this flow indicate eosinophilic airway inflammation and the cut-off can be applied to detect asthma in symptomatic individuals [7,8].

F_{ENO} measurement at multiple flows are used to estimate the anatomical origin of NO in exhaled air [9–12]. A simple two compartment model of the airways has been adopted [6] to define the alveolar NO concentration (C_{ANO}), maximum airway NO flux (J'_{awNO}) and airway NO diffusion (D_{awNO}) [13]. These estimates have provided extended information in subjects with asthma [12–15].

In healthy subjects, a fraction of F_{ENO} seems to originate in the upper airways and oropharynx, partly due to bacterial production of NO and dietary intake of nitrate [16–21].

Rinsing the oral cavity with carbonated water lowers F_{ENO} levels significantly in healthy and asthmatic subjects [22,23]. Although the ATS/ERS guidelines suggest that a mouthwash before F_{ENO} measurements may reduce oral F_{ENO}, it is not part of the standardized clinical procedure [5], but only recommended in physiological research [6]. However, rinsing of the mouth with carbonated water has been routinely adopted in our laboratory at the Helsinki University Hospital prior to F_{ENO} measurements since 1995.

The aim of this study was to compare the effects of a carbonated water mouthwash and a tap water mouthwash on F_{ENO} at different flow rates, as well to analyse the effects of the mouthwashes on C_{ANO}, J'_{awNO} and D_{awNO}. Furthermore, we aimed to investigate the repeatability of the F_{ENO} measurements.

Methods

We recruited 30 volunteers aged 16–68 years, either healthcare workers ($n = 21$) or patients ($n = 9$). The patients

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enrolled have had asthmatic symptoms and were previously referred for F_{ENO} testing, either to the Laboratory of Clinical Physiology at the University Hospital in Helsinki or the Skin and Allergy Hospital in Helsinki. Healthcare workers who volunteered were included without further selection. None of the subjects were excluded. All participants were asked to fill a questionnaire including questions regarding smoking habits, asthma, allergic rhinitis and COPD.

Nine participants reported having allergic rhinitis, six asthma and one COPD. Two participants smoked regularly, one less than five cigarettes a day, the other from 5 to 15 a day. From available medical records, we searched for respiratory or systemic diseases that could influence F_{ENO} values, reason for referral to F_{ENO} testing and current medication. Nine participants had a chronic respiratory disease or respiratory symptoms. Among these were four patients with asthma, three with respiratory symptoms due to building dampness but low or negative bronchial hyperreactivity, one with eosinophilic bronchitis and another with Sjögren's syndrome. The patient with Sjögren's syndrome had no interstitial lung disease, previously ruled out through a high resolution computed tomography. Six participants had prescriptions for short acting beta$_2$-agonists, four used inhaled corticosteroids regularly, two used antihistamines and one used leukotriene receptor antagonists. Spirometric data were available in 25 subjects. None of these subjects had current bronchodilator reversibility (defined as increase in FEV1 or FVC over 12% and 200 mL) [24]. Demographic, anthropometric and spirometric data are summarised in Table 1.

The F_{ENO} measurements were made either at the Skin and Allergy Hospital or at the Finnish Institute of Occupational Health, both in Helsinki. The NO analysers used were chemiluminescence CLD 88 sp and the devices were EXHALIZER®'s D with SPIROWARE® software from Eco Medics AG (Dürnten, Switzerland) and calibrated according to the manufacturer's instructions by using certified span gas (AGA Gas BV, Amsterdam, Netherlands) and NO free air by using a zero-air filter (DENOX 88 unit). The inspired gas was NO free (<5 ppb, maximum recorded fractional inspired F_{ino} was 3.1 ppb). Before the measurements, the ultrasonic flow sensor was calibrated with a calibration syringe (Hans Rudolph Inc., Shawnee, KS). F_{ENO} measurements for each subject were performed during two consecutive days. The flowchart in Figure 1 visualizes the order of the procedures. The measurements were made at four different expiratory flow rates V′ (30, 50, 100 and 300 mL/s). The sequence of the flow rates was kept the same, starting with a 50 mL/s flow rate, followed by 30, 100 and 300 mL/s. The mouthwash procedure was defined as rinsing the oral cavity for 30–60 seconds with 100 mL of tap water or carbonated water. On the first day, the subjects performed a mouthwash with tap water before the measurements at the first flow level, and then repeated the mouthwash with tap water before measuring at every flow level. After reaching the highest flow level, i.e. 300 mL/s, there was a 15 min pause before starting a new array. After this time interval, all measurements were repeated, but 100 mL of carbonated water was used to perform the mouthwash. During the second consecutive day, all tests were repeated in the same fashion (i.e. two multiple-flow measurements of F_{ENO} with different mouthwashes, totalling two arrays of testing on each day). The mouthwashes were not randomized, since the carbonated water mouthwash has a significantly longer effect on F_{ENO} than the tap water mouthwash [22].

A minimum of two measurements of F_{ENO} at each flow were performed to obtain an acceptable value. The measurements at each stage were accepted if its variation was no more than 2 ppb. If more than two attempts were needed for getting a valid measurement, the oral rinsing was repeated. To further analyse the data, a mean value was obtained from four single determinations (two obtained each day) for every subject at each flow and mouthwash setting.

Table 1. Demographic, anthropometric and spirometric data of the study participants.

	n	Mean (SD)	Range	Spirometry	*n*	Mean (SD)	Range
Age (years)	30	43 (14)	16–68	FVC (l)	25	4.1 (1.1)	2.65–6.69
Height (cm)	30	171 (8)	157–186	FVC (%[a])	25	93 (16)	50–120
Weight (kg)	30	73 (15)	49–109	FEV1 (l)	25	3.3 (0.9)	2.06–5.36
Female/male	17/13	57%/43%		FEV1 (%[a])	25	93 (16)	50–117
Asthma	6	21%		FEV1/FVC ratio	25	0.80 (0.1)	0.69–0.98
Allergic rhinitis	9	31%		FEV1/FVC (%[a])	25	98 (7)	88–125
Smoking	2	7%					
COPD	1	3%					

Data presented as mean (SD) or percentage of total case number.
[a]Percentage of predicted value (predicted according to Viljanen et al. [25]).

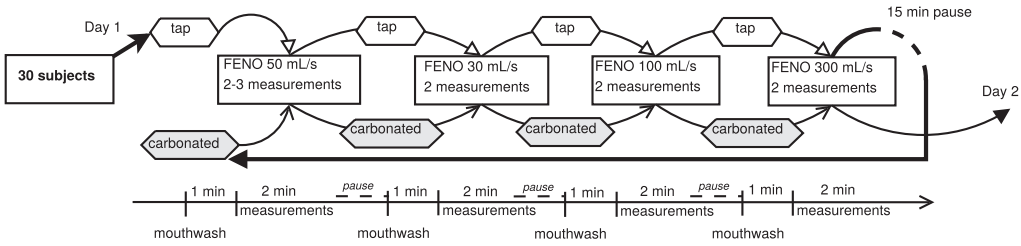

Figure 1. Flowchart illustrating the procedures, mouthwashes and repetitions performed during one day. Tap: tap water mouthwash, carbonated: carbonated water mouthwash.

The bottled carbonated water used for the mouthwash was commercially available (HARTWALL VICHY ORIGINAL®, Oy Hartwall Ab, Helsinki, Finland) and had a declared pH of 5.7–5.9; the tap water used had a pH of 8.3. More detailed information regarding the solutions can be found under Lassmann-Klee et al. [22].

All recommendations according to ATS/ERS were followed, except from the selection of a wide range of expiratory flows and using a mouthwash [5]. All subjects refrained from smoking four hours, from drinking coffee two hours and from eating and drinking one hour before the study. Also strenuous exercising prior measuring was discouraged.

Ethical committee approval was received (99/13/03/00/15) and we followed the ethical principles stated in the Declaration of Helsinki [26].

Statistics

Analyses were performed using IBM® SPSS® statistics software version 22 (Armonk, NY), RKWARD® version 0.6.5 frontend and RSTUDIO® version 1.1.383 frontend to the R statistics language (THE R FOUNDATION®, Vienna, Austria), and we partially used GRAPHPAD® PRISM® version 5.04 to obtain the graphs (La Jolla, CA). The comparison of F_{ENO} was made using a Wilcoxon test for paired probes. We accepted a significance level of $\alpha = 0.05$ as significant. The mean (SD) was obtained for each subject from four F_{ENO} measurements after each mouthwash at flows 30, 50, 100 and 300 mL/s, respectively. The median and the median absolute deviation (MAD) was calculated too [27]. To prove the accuracy of the measurements, we used the coefficient of variation (c_v), defined as the quotient of standard deviation (SD) and mean. We defined a total mean value of $c_v <$ 10% as acceptable. C_{ANO}, J'_{awNO} and D_{awNO} were calculated using a nonlinear logarithmic transformation (we used starting estimated values of the quadratic T transformation, a second order approximation) according to Eckel et al. [11] using following equation:

$$\log F_{ENO} = \log\left(\frac{J'_{awNO}}{D_{awNO}} + \left(C_{ANO} - \frac{J'_{awNO}}{D_{awNO}}\right)^{\frac{-D_{awNO}}{V}}\right) + \epsilon \quad (1)$$

C_{ANO}, J'_{awNO} or D_{awNO} were also calculated using the Högman and Meriläinen algorithm (HMA), with mean F_{ENO} and mean V' values for the flow rates: 30 mL/s, 100 mL/s and 300 mL/s [12]. Cases with negative values for C_{ANO} and with failure of consistency check were not ruled out.

The comparison of C_{ANO}, J'_{awNO} or D_{awNO} between mouthwashes was made using a Wilcoxon test for paired probes. All target variables were not normally distributed (Shapiro–Wilk's test). The differences between c_v were analysed with a Wilcoxon test when comparing mouthwashes. A comparison of all calculated c_v results was made with Friedman's two-way ANOVA.

Results

The results of F_{ENO} measurements at multiple flow levels are listed in Table 2 and visualised in Figure 2. Individual F_{ENO} values are visualised in Figure 3. F_{ENO} ranged between 4.4 and 221.6 ppb and the median was 14.94 (MAD: 6.76) ppb at a flow rate of 50 mL/s after rinsing with tap water. The mouthwash with carbonated water reduced F_{ENO} significantly compared to the tap water mouthwash at every flow rate.

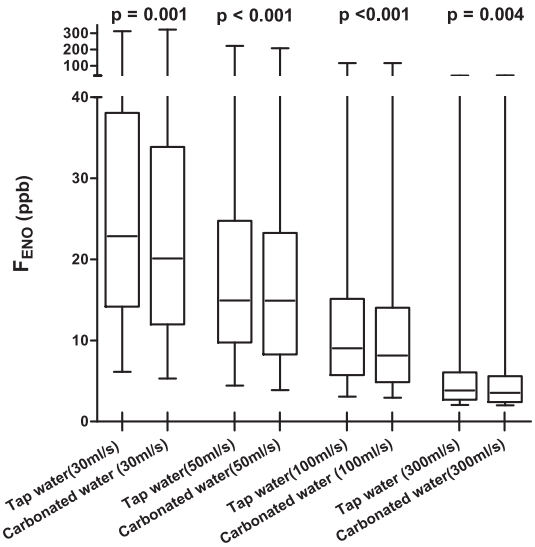

Figure 2. F_{ENO} measured at multiple expiratory flow levels after different mouthwashes. Pairs compared with the Wilcoxon signed-rank test.

Table 2. Summarized results of F_{ENO} at every flow rate and mouthwash either with tap water or carbonated water.

F_{ENO} (ppb)	Median	MAD	IQR	p^a	Mean (SD)	Difference[b]	Mean c_v[c]
Flow 30 mL/s, tap water	22.86	10.11	20.4	.001	39.21(57.04)	−4.6%	8.6 (4.5)
Flow 30 mL/s, carbonated water	20.11	9.49	20.7		37.40(59.28)		7.3 (5.0)
Flow 50 mL/s, tap water	14.94	6.76	13.3	<.001	27.27(40.19)	−6.4%	8.4 (6.2)
Flow 50 mL/s, carbonated water	14.91	7.24	14.4		25.51(38.01)		6.6 (3.9)
Flow 100 mL/s, tap water	9.03	3.91	8.8	<.001	15.41(21.58)	−4.4%	7.0 (6.6)
Flow 100 mL/s, carbonated water	8.15	3.68	8.7		14.74(21.72)		6.1 (4.0)
Flow 300 mL/s, tap water	3.84	1.36	3.2	.004	6.03(7.29)	−4.2%	6.9 (4.1)
Flow 300 mL/s, carbonated water	3.53	1.31	3.0		5.77(7.45)		8.0 (5.3)

Data presented as median, median absolute deviation (MAD), interquantile range (IQR), $n = 30$. Mean c_v calculated from individual c_v of F_{ENO} (consisting of at least four valid F_{ENO} results for each flow rate and mouthwash).
[a]Wilcoxon's signed ranks test.
[b]Relative decrease of the mean.
[c]Presented as mean (SD) in percent.

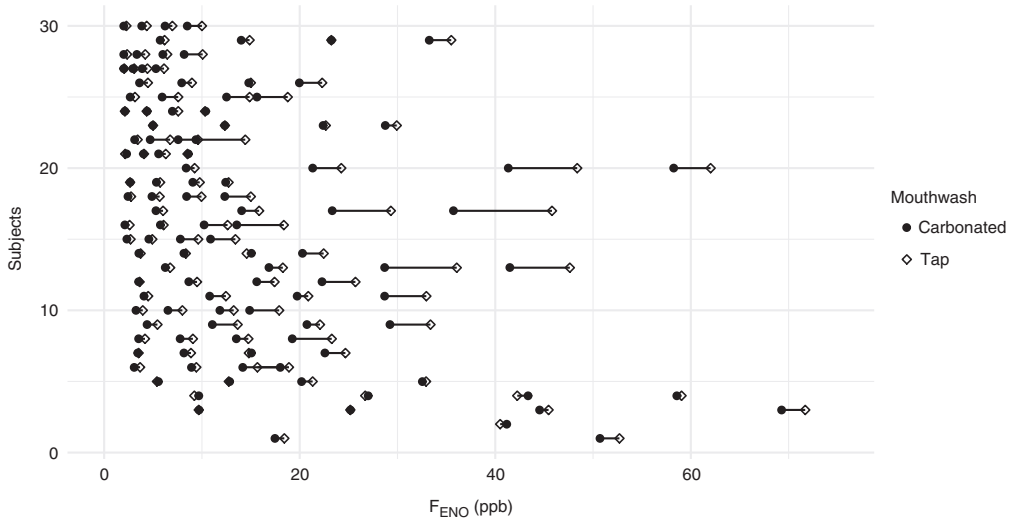

Figure 3. Individual F_{ENO} values measured at multiple expiratory flow levels after different mouthwashes. Ordinate represents the individuals ($n = 30$), abscissa represents F_{ENO} (ppb) at multiple expiratory flow levels after: carbonated water mouthwash (black dots), and tap water mouthwash (grey rhombi). Abscissa truncated at 75 ppb. Subjects 1 and 2 had higher F_{ENO} (ppb) values (not shown). The missing higher values (tap water/carbonated) for subject 1 are: 128/128, 85/79; and for subject 2 are: 311/322, 222/208, 117/117.

Table 3. Results of J'_{awNO}, C_{ANO} and D_{awNO}, after carbonated water or tap water mouthwash.

	J'_{awNO} (pL/s)	p^a	C_{ANO} (ppb)	p^a	D_{awNO} (pL·s^{-1}·ppb^{-1})	p^a
Tap water	836.36 (457.58)	.015	0.88 (0.48)	.715	14.19 (7.52)	.299
Carbonated water	829.97 (443.12)		0.92 (0.33)		18.60 (3.87)	
Tap water[b]	796.88 (449.86)	<.001	1.00 (0.39)	.598	15.63 (6.95)	.871
Carbonated water[b]	724.96 (392.60)		1.04 (0.30)		15.28 (3.67)	

Data presented as median (median absolute deviation), $n = 30$.
[a]Wilcoxon's signed ranks test.
[b]Calculated with Högman and Meriläinen algorithm.

The mean c_v of F_{ENO} stayed under 10% for all flow levels. Comparing the c_v between tap water rinsing and carbonated water rinsing, no significant difference was found at any flow level (at 30 mL/s $p = .094$; at 50 mL/s $p = .125$; at 100 mL/s $p = .245$; at 300 mL/s $p = .688$). Analysing c_v for all flow levels and mouthwashes resulted in no difference ($p = .202$).

When comparing J'_{awNO} between mouthwashes, the median J'_{awNO} for carbonated water was significantly lower using both models. C_{ANO} and D_{awNO} did not differ significantly between both mouthwashes. These results are summarized in Table 3.

The NO fraction of inspired gas was at all times below 20 ppb as recommended, having a mean value of 0.35 ppb (SD 0.34) [5].

Discussion

Mouthwashes

We found a statistically significant difference in F_{ENO} between mouthwashes at all expiratory flow levels. F_{ENO} was statistically significantly lower after rinsing with carbonated water compared to tap water at all expiratory flow levels. This confirms previous studies in which carbonated water was used to perform a mouthwash [22,23], and suggests endorsement of a carbonated water mouthwash prior multiple-flow testing.

In the present study, the difference between mouthwashes was ca. −5% at all flow levels. This decrease of F_{ENO} between mouthwashes equals our previously observed reduction. We demonstrated recently that both carbonated water and tap water mouthwashes lower F_{ENO} significantly compared with the baseline and observed an immediate decrease of F_{ENO} after a carbonated water mouthwash of −18% and of −13% after a tap water mouthwash [22]. Additionally, Piirilä et al. [23] found at 50 mL/s a decrease from baseline (without mouthwash) of ca. −10% after a carbonated water mouthwash in a healthy population. In comparison, previous multiple flow studies have found a relative decrease in F_{ENO} of ca. −10% after a chlorhexidine mouthwash in children and adolescent (both asthmatic and healthy) [28] and of ca. −15% in healthy adults [29].

Furthermore, the tap water mouthwash's effect is short-lasting, only two minutes. On the other hand, the carbonated water mouthwash's effect is longer, lasting 12 minutes, and more effective if compared with tap water [22]. For this

reason, we did not randomize the subjects in the present study and performed the measurements with tap water first.

Our data were accurately collected, since the intra-individual c_v of F_{ENO} stayed low and the mean c_v below 10%. We did not find a difference in c_v between mouthwashes. The c_v at 100 mL/s is similar to previously reported by Ekroos et al. [30] for a healthy male population taking into account a time period of maximal 24 hours.

Multiple flow F_{ENO} and mouthwashes

As we expected, J'_{awNO} was significantly lower after the carbonated water mouthwash compared to the tap water mouthwash. There was no statistically significant difference between mouthwashes when analysing C_{ANO} and D_{awNO}. We verified this results with estimated values for J'_{awNO}, C_{ANO} and D_{awNO} and two different models. This further strengthens our result, that the carbonated water mouthwash affects the airway fraction (due to oral NO reduction) and not the peripheral alveolar NO, neither the NO diffusion. We could state the hypothesis that the carbonated water rinsing provides more exact values (without oral contamination) in general. Heijkenskjöld-Rentzhog et al. published similar findings when analysing J'_{awNO} and C_{ANO} after a chlorhexidine mouthwash [28]. This is in contrast to results by Malinovschi et al. [29] who found also a decrease in C_{ANO} after a chlorhexidine mouthwash. Both used a trumpet-shaped model with corrections for axial diffusion (TMAD). Kerckx et al. [31] demonstrated that, when using a model correcting for axial diffusion to estimate C_{ANO} in asthmatic patients (unobstructed and well-managed), C_{ANO} is normal, even when J'_{awNO} is elevated. This has been confirmed also for patients with severe asthma and during exacerbation [32].

For our purposes, we employed a two compartment model of the lung [4], without considering axial diffusion [9], and applied a robust mathematical model [11]. This model was tested previously exactly at the same flow levels by Eckel et al. and did not impose flow limitations unlike other models [11]. We obtained also values using the HMA (without axial diffusion) and the results between mouthwashes were similar. Although results from models with and without axial diffusion are not directly equivalent, an analogy can be made between the main findings of different studies.

The strength of our study was using different parameters to examine the effect of the carbonated water mouthwash and for that using two different mathematical models. Our calculations with Equation (1) provided negative values for C_{ANO} in five cases for tap water mouthwash and four cases for carbonated water mouthwash, which were not excluded. A similar amount of negative values were obtained with the HMA. This has been a regular problem with other models providing a greater proportion of negative concentration values. We disregarded the importance of these data, since the comparison of C_{ANO} between mouthwashes showed no difference (even when replacing these values for zero). Our extended analysis of J'_{awNO}, C_{ANO} and D_{awNO} found these values comparable to previous results in a healthy population [33]. All results for C_{ANO} were under 2.3 ppb and none of the subjects had a medical condition associated with interstitial lung disease, therefore we assume that the subjects with measured F_{ENO} over 25 ppb ($n = 7$) had an elevated airway production of NO.

Limitations and implications

We acknowledge the limitation of not randomizing the order of the expiratory flows levels. However, the limitation was imposed by the nature of the flow levels used, i.e. the high flow representing an expiratory burden to the participants. Previous studies reported a decrease in F_{ENO} after forced expirations [34–36]. We selected on purpose an incremental flow order to avoid the high flow expiration influencing the slower expiration manoeuvres.

The tap water used to compare the effect of carbonated water had a pH value over 8. The tap water in European countries has a pH of 6.5–9.5 units (Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption) and this range also complies with WHO guidelines. Many countries may have lower tap water's pH values than 8. This may enhance the tap water's effect on oral F_{ENO} and lower the differences between tap water and carbonated water. Nevertheless, further studies are needed to elucidate if the pH is the crucial factor of the mouthwash solutions.

Carbonated water poses an ideal candidate for performing a mouthwash before multiple flow measuring. It eliminates oral NO interference more effectively than tap water and for a prolonged period of time. Our recent publication [22] argues for the clinical application of a mouthwash before F_{ENO} measurement. Here, we demonstrate it influences only the airway fraction of F_{ENO}, and using carbonated water as a mouthwash, in a more pronounced way. Probably one mouthwash procedure with carbonated water may suffice when performing a routine multiple-flow investigation, but repetitions might be useful if exact values are needed, e.g. in physiological research.

Conclusions

We conclude that a carbonated water mouthwash can significantly reduce oropharyngeal NO compared to a tap water mouthwash at expiratory flows of 30–300 mL/s without affecting the C_{ANO} nor the D_{awNO}. We imply that a carbonated water mouthwash is suitable for routine multiple-flow F_{ENO}-analysis and evidently useful in clinical research. This study strengthens the view that mouthwash procedures shall be taken into account when comparing F_{ENO} values.

Acknowledgements

We thank the staff members: Sari Fischer, Helena Punkari and Elina Voutilainen for performing the F_{ENO} measuring and also Tommi Pallasaho for data collection.

Disclosure statement

No conflicts of interest are declared by the author(s).

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Funding

This work was supported by the Nummela Sanatorium Foundation (PP 2015, 2017) (AS 2016), Finnish State Funding for University-Level Health Research (TYH: 2013354), The Research Foundation of the Pulmonary Diseases (PLK 2017, 2018), Tampere Tuberculosis Foundation: Eero Hämäläinen (PLK 2017, 2018), Ida Montin Foundation (PLK 2017), Väinö and Laina Kivi Foundation (PLK 2017, 2018) and the Nordic Council of Ministers: NordForsk.

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Clin Physiol Funct Imaging (2019) **39**, pp315–321 doi: 10.1111/cpf.12574

Converting F_{ENO} by different flows to standard flow F_{ENO}

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Accepted for publication
Received 5 June 2018;
accepted 30 April 2019

Key words
adults; alveolitis; asthma; children; COPD; fractional exhaled nitric oxide (F_{ENO}); mouthwash; multiple-flow

Abstract

In clinical practice, assessment of expiratory nitric oxide (F_{ENO}) may reveal eosinophilic airway inflammation in asthmatic and other pulmonary diseases. Currently, measuring of F_{ENO} is standardized to exhaled flow level of 50 ml s^{-1}, since the expiratory flow rate affects the F_{ENO} results. To enable the comparison of F_{ENO} measured with different expiratory flows, we firstly aimed to establish a conversion model to estimate F_{ENO} at the standard flow level, and secondly, validate it in five external populations. F_{ENO} measurements were obtained from 30 volunteers (mixed adult population) at the following multiple expiratory flow rates: 50, 30, 100 and 300 ml s^{-1}, after different mouthwash settings, and a conversion model was developed. We tested the conversion model in five populations: healthy adults, healthy children, and patients with COPD, asthma and alveolitis. F_{ENO} conversions in the mixed adult population, in healthy adults and in children, showed the lowest deviation between estimated $\hat{F}_{ENO}$ from 100 ml s^{-1} and measured F_{ENO} at 50 mL s^{-1}: −0·28 ppb, −0·44 ppb and 0·27 ppb, respectively. In patients with COPD, asthma and alveolitis, the deviation was −1·16 ppb, −1·68 ppb and 1·47 ppb, respectively. We proposed a valid model to convert F_{ENO} in healthy or mixed populations, as well as in subjects with obstructive pulmonary diseases and found it suitable for converting F_{ENO} measured with different expiratory flows to the standard flow in large epidemiological data, but not on individual level. In conclusion, a model to convert F_{ENO} from different flows to the standard flow was established and validated.

Introduction

Chronic bronchial inflammation of the respiratory mucosa can lead to bronchial hyperreactivity and airway obstruction. Clinicians often employ fractional exhaled nitric oxide (F_{ENO}) to evaluate bronchial eosinophilic inflammation (NICE, 2017). F_{ENO} values are flow-dependent, and an expiratory flow rate of 50 ml s^{-1} mirrors the bronchial nitric oxide (NO) production and not the NO with peripheral origin (Tsoukias & George, 1998; Högman *et al.*, 2000). For this reason, F_{ENO} measurement is currently standardized at the expiratory flow rate of 50 ml s^{-1} (ATS/ERS, 2005, Horváth *et al.*, 2017). Prior to the standardization, F_{ENO} was acquired in Northern Europe with expiratory flow rates of 50-300 ml s^{-1} (Högman *et al.*, 1997; Ekroos *et al.*, 2002; Rouhos *et al.*, 2008) and a previous guideline endorsed the use of flow rates between 167 and 250 ml s^{-1} (Kharitonov *et al.*, 1997). Many pioneers in F_{ENO} investigation adopted a flow rate of 100 ml s^{-1} (Kharitonov & Barnes, 2001). Unfortunately, data measured at different flow levels have been difficult to compare, since F_{ENO} values are affected by the flow rate used and represent NO from anatomically different lung parts. Therefore, a conversion method to interpolate F_{ENO} values to equivalent F_{ENO} values at diverse flows was needed. Since the lowering effect of mouthwashes on F_{ENO} values is well documented (Lassmann-Klee *et al.*, 2018a,b), the conversion method should address also the mouthwashes. The aim of this study was to establish a method for converting F_{ENO}, measured at different expiratory flow levels, to the standard F_{ENO} measured at 50 ml s^{-1} and validate this method. Further on, we aimed to determine the need of considering the mouthwashes in the conversion method.

Glossary

F_{ENO}, Fractional exhaled nitric oxide
$\hat{F}_{ENO}$, Estimated fractional exhaled nitric oxide
$\dot{V}$, Expiratory flow rate
NO, Nitric Oxide

Methods

Data acquisition

We recruited 30 healthy or asthmatic adults as volunteers (henceforth referred as 'mixed adult population') to develop a conversion method. We have previously described this population (Lassmann-Klee *et al.*, 2018b). The volunteers were adult patients (n = 9) or healthcare workers (n = 21). The patients invited were previously referred for F_{ENO} assessment to the Laboratory of Clinical Physiology or to the Skin and Allergy Hospital at the Helsinki University Central Hospital area. The healthcare employees were included in the study without exclusions. The patients enrolled had respiratory symptoms or a chronic respiratory disease, including asthma (n = 4), eosinophilic bronchitis (n = 1), building-related respiratory symptoms (n = 3) and Sjögren's syndrome (n = 1). Spirometric data (n = 25) were analysed, and none of the participants had actual bronchodilator reversibility (Pellegrino *et al.*, 2005).

F_{ENO} measurements were performed at the Finnish Institute of Occupational Health and at the Skin and Allergy Hospital with CLD 88 sp chemiluminescence NO analysers and EXHALIZER®'s D devices using SPIROWARE® software (Eco Medics AG, Switzerland). The devices were calibrated in compliance with the producer's specifications: use of certified span gas (AGA Gas BV, Amsterdam, Netherlands) and a zero-air filtering system (DENOX 88 unit). Additionally, a calibration syringe (Hans Rudolph Inc., USA) was used to calibrate the ultrasonic flow sensor. We complied with all advices from the ATS/ERS statement (ATS/ERS, 2005).

We performed F_{ENO} measurements in our mixed adult population (n = 30) from September 2016 until May 2017, and the tests for each volunteer were scheduled on 2 consecutive days. All the 30 volunteers followed a mouthwash protocol with tap water and carbonated water. Detailed description of the mouthwashes' protocol is available in our recent study (Lassmann-Klee *et al.*, 2018b). Briefly, the F_{ENO} measurements were performed after a mouthwash with 100 ml of tap water at each flow level. After 15 min, all measurements were repeated after a mouthwash with 100 ml of carbonated water at each flow level. The mouthwashes' effect, duration and chemical composition are well documented (Lassmann-Klee *et al.*, 2018a,b).

Secondly, we selected 10 healthcare workers from the aforementioned volunteers to perform an additional measurement phase. The selection criterion was inclusion only of those employed at the Skin and Allergy Hospital. In the third appointments, the 10 healthcare workers performed the measurements without a mouthwash.

F_{ENO} was acquired from all participants at the following multiple expiratory flow rates: 50, 30, 100 and 300 ml s^{-1}. At least two measurements of F_{ENO} were obtained at each flow level. The values were accepted, if its variation was less than 2 ppb.

Validation

For validating our conversion method, 5 different datasets of previously published articles acquired at the Tampere University Hospital were available. They contained multiple-flow data from 69 healthy adults (Lehtimäki *et al.*, 2010a,b), 66 healthy children (Sepponen *et al.*, 2008), 74 steroid-naive adults with COPD (Lehtimäki *et al.*, 2010a), 40 steroid-naive adults with asthma (Lehtimäki *et al.*, 2001) and 17 subjects with untreated alveolitis (Lehtimäki *et al.*, 2001). The validation process is explained in the statistical section.

This study followed the ethical principles of the declaration of Helsinki (World Medical Association Declaration of Helsinki, 2013) and received approval from an ethical committee (99/13/03/00/15). All participants signed an informed consent.

Statistics

Modelling the conversion method

Analyses were performed using RSTUDIO® version 1·1·383 frontend to the R statistics language (R Core Team, 2018). We agreed on a significance level of $\alpha = 0{\cdot}05$ as significant. We calculated the arithmetic mean from individual F_{ENO} values obtained at each flow level. The mean values were plotted against the expiratory flow rate $\dot{V}$ in a double logarithmic scale, and we performed a non-linear regression. We obtained a slope and intercept and analysed the regression line to develop our conversion model. To further refine the model, we acquired a non-linear least squares estimation of the non-linear model parameters. This model was used to estimate $\hat{F}_{ENO}$ values from F_{ENO} values measured at different flow rates.

Validation

To test the validity of our model, we converted F_{ENO} values measured at 30, 100 and 300 ml s^{-1} to estimated $\hat{F}_{ENO}$ values for a standard flow rate of 50 ml s^{-1}. Afterwards, we compared the estimated $\hat{F}_{ENO}$ values to the actual F_{ENO} measured at 50 ml s^{-1}. To assess the agreement between estimated $\hat{F}_{ENO}$ and measured F_{ENO}, we performed an analysis (see below) according to Bland & Altman (2010). Further on, the correlation coefficient rho was obtained with Spearman's formula to investigate linearity.

To validate our conversion model in different external populations, we compared the estimated $\hat{F}_{ENO}$ converted from 100 ml s^{-1} with F_{ENO} measured at 50 or 40 ml s^{-1}. For this external validation, a method described by Bland & Altman (2010) was employed. Accordingly, we obtained the individual differences of F_{ENO}, the mean of differences (bias) and the 1·96 standard deviations of the mean (95% limits of agreement).

Additionally, we performed a linear regression analysis (glm) between F_{ENO} values measured at 50 ml s^{-1} after the tap water and carbonated water mouthwashes, to obtain a

relation between the mouthwashes and to provide an additional equation to convert measurements with these two mouthwashes to the standard flow level (50 ml s^{-1}).

When necessary, raw data were examined for outliers using the absolute deviation around the median (3 deviations as threshold). If cases were omitted, the conversion was repeated and the differences and level of agreements adjusted (Leys *et al.*, 2013).

Results

Conversion model

We plotted the mean F_{ENO} values against the expiratory flow rate $\dot{V}$ and performed a non-linear regression. Acquiring non-linear least squares parameter estimates resulted in a slope of −0·8416 SE(0·3192) for carbonated water, a slope of −0·84 SE(0·2989) for tap water and a slope of −0·83111 SE (0·05424) in the absence of a mouthwash. In the latter case, the equation model can be further defined as:

$$\hat{F}_{ENO} = k \cdot \dot{V}^{-0.83111} \quad (1)$$

Plotting our model with Eq. using measured F_{ENO} and $\dot{V}$, as well as calculated values for k, resulted in Fig. 1.

The linear regression of F_{ENO} at 50 ml s^{-1} after a tap water mouthwash in relation to carbonated water resulted in a slope coefficient of 1·055 ppb and intercept of 0·354 ppb (P<0·001).

When employing the different estimating slopes for the $\hat{F}_{ENO}$ conversions with tap water and carbonated water mouthwashes, the mean estimated $\hat{F}_{ENO}$ for the carbonated water mouthwash was ca. −4·5% lower than the mean estimated $\hat{F}_{ENO}$ for tap water at all flow levels (unadjusted).

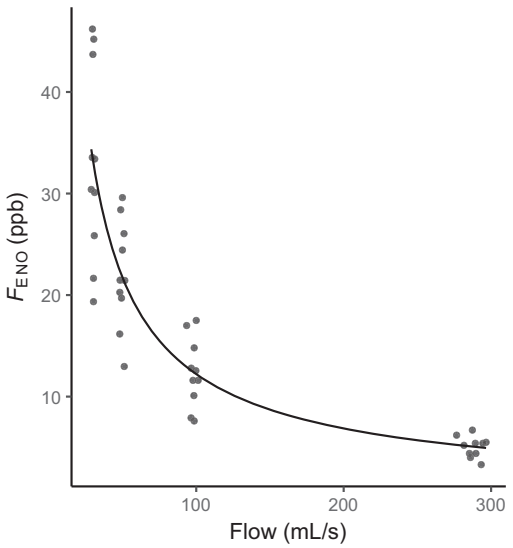

Figure 1 F_{ENO} as a function of expiratory flow (without mouthwash), n = 10. Curve shows the equation $\hat{F}_{ENO} = k \cdot \dot{V}^{-0.83111}$.

Validation results in mixed adult population

Using Eq. 1, we calculated the values for $\hat{F}_{ENO}$ (flow level 50 ml s^{-1}) interpolated from data obtained at 100 ml s^{-1}. Applying the (Bland & Altman, 2010) method resulted in mean (SD) differences between the estimated $\hat{F}_{ENO}$ (flow level 50 ml s^{-1}) and the measured F_{ENO} (flow level 50 ml s^{-1}) of -0·45(2·44) ppb, upper 95% limit of agreement of 4·34 ppb and lower 95% limit of agreement of −5·23 ppb. The measured F_{ENO} and the estimated $\hat{F}_{ENO}$ had a good correlation (Spearman's ρ = 0·87; P<0·0001).

We also estimated $\hat{F}_{ENO}$ (50 ml s^{-1}) from values measured at all flow levels and mouthwash settings. All differences with the (Bland & Altman, 2010) method showed a good agreement, and the total unadjusted mean of the absolute deviation of $\hat{F}_{ENO}$ from F_{ENO} was 0·72 ppb. All estimated values were highly correlated with corresponding measured values. Table 1 summarizes these results. Figure 2 exemplifies the unadjusted mean differences of $\hat{F}_{ENO}$ and F_{ENO} after applying Eq. 1 (conversion with carbonated water mouthwash from flow of 100 ml s^{-1}). After adjusting measured F_{ENO} by removing outliers and performing a new estimation, a better agreement was found between estimated $\hat{F}_{ENO}$ and measured F_{ENO}, and total mean of the absolute deviations of $\hat{F}_{ENO}$ from F_{ENO} was 0·66 ppb. The adjusted results after controlling for outliers can be also found in Table 1.

Validation results in external populations

With the same approach, we converted F_{ENO} data obtained at 100 ml s^{-1} (Lauri Lehtimäki *et al.*, 2001; Sepponen *et al.*, 2008; Lehtimäki *et al.*, 2010a,b) to estimated $\hat{F}_{ENO}$ (flow level 50 or 40 ml s^{-1}) without a mouthwash (Eq.). The mean difference between estimated $\hat{F}_{ENO}$ and measured F_{ENO} was lowest (0·27 ppb) in the healthy children group, followed by the healthy adult group (−0·44 ppb), as shown in Fig. 3. The mean difference illustrated in Fig. 2 of steroid-naive adults with asthma was −1·68 ppb. In Fig. 4, the mean difference shown is −1·16 ppb in steroid-naive adults with COPD, and 1·47 in the untreated alveolitis population. The healthy groups had narrow limits of agreement, in contrast to the groups with diseases. Table 2 synthesizes these results. Additionally, Fig. 5 demonstrates the distribution of the differences in all populations. Table 3 contains the correlation between the measured and estimated F_{ENO} values and provides information concerning the linearity between the values.

Discussion

Conversion model

We found that using a non-linear regression yielded a simple model to convert F_{ENO} values measured at different flows to estimated $\hat{F}_{ENO}$ at 50 ml s^{-1}. To prove the feasibility of the equation, we compared estimated $\hat{F}_{ENO}$ levels at the standard

Table 1 Bland–Altman statistics in our mixed healthy and asthmatic adult population (n = 30) and in healthcare workers (n = 10) with mean, bias[a], levels of agreement and standard deviation (SD) of the differences between estimated $\hat{F}_{ENO}$ from different flow levels and mouthwashes, and measured F_{ENO} at 50 ml s^{-1} (tap water: 27·27 ppb; carbonated water: 25·51 ppb; no mouthwash: 22·05)

Mean estimated $\hat{F}_{ENO}$ (ppb) at 50 ml s^{-1} from flow level and mouthwash		Level of agreement				Adjusted values Level of agreement					
		Bias[a]	Lower	Upper	SD	bias[a]	Lower	Upper	SD	rho	[b]
30 ml s^{-1}; tap	25·24	−2·03	−11·17	7·10	4·66	−1·23	−5·44	3·0	2·15	0·96	3
100 ml s^{-1}; tap	26·99	−0·28	−7·42	6·86	3·64	−0·11	−3·67	3·44	1·81	0·98	3
300 ml s^{-1}; tap	26·27	−1·00	−19·02	17·01	9·19	0·74	−5·79	7·27	3·33	0·95	2
30 ml s^{-1}; carbonated	24·23	−1·28	−4·92	2·36	1·86	−1·50	−4·90	1·90	1·73	0·99	3
100 ml s^{-1}; carbonated	25·65	0·13	−4·28	4·55	2·25	−0·08	−3·32	3·16	1·65	0·99	4
300 ml s^{-1}; carbonated	25·07	−0·44	−13·32	12·43	6·57	0·99	−4·69	6·67	2·90	0·95	4
30 ml s^{-1}; no mouthwash	21·64	−0·41	−5·89	5·06	2·79	−0·41	−5·89	5·06	2·79	0·84	0
100 ml s^{-1}; no mouthwash	21·60	−0·45	−5·23	4·34	2·44	−0·45	−5·23	4·34	2·44	0·87	0
300 ml s^{-1}; no mouthwash	21·62	−0·43	−5·67	4·82	2·68	−0·43	−5·67	4·82	2·68	0·82	0

Raw data and adjusted values for outliers. Rho according to Spearman's test.
[a]average of the differences between estimated $\hat{F}_{ENO}$ and measured F_{ENO}.
[b]Number of observations excluded with the adjustment.

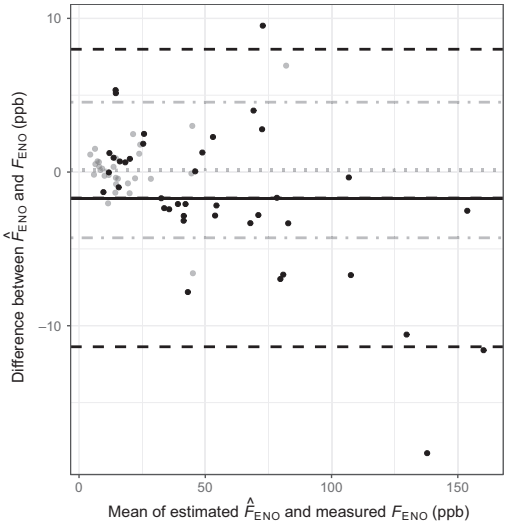

Figure 2 Bland–Altman plot with mean of measured F_{ENO} and estimated $\hat{F}_{ENO}$ from 100 ml s^{-1} in asthmatics (grey dots, n = 40) and our mixed adult population (black dots, n = 30), plotted against the differences in F_{ENO}. In asthmatics: mean differences (grey dotted line), 1·96 standard deviations (grey dot-slashed line). In mixed adult population: mean differences (black solid line), 1·96 standard deviation (black slashed line). In asthmatics F_{ENO} measured at 40 ml s^{-1}. In mixed adult population F_{ENO} measured at 50 ml s^{-1} after carbonated water mouthwash.

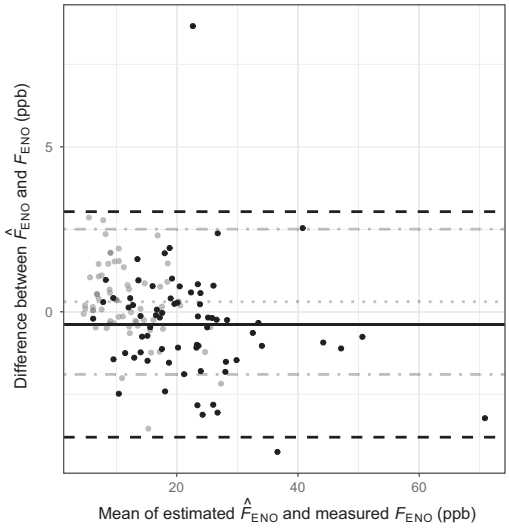

Figure 3 Bland–Altman plot with mean of F_{ENO} measured at 50 ml s^{-1} and estimated $\hat{F}_{ENO}$ from 100 ml s^{-1} in healthy children (grey dots, n = 66) and in healthy adults (black dots, n = 69), plotted against the differences in F_{ENO}. In healthy children: mean differences (grey dotted line), 1·96 standard deviations (grey dot-slashed line). In healthy adults: mean differences (black solid line), 1·96 standard deviation (black slashed line).

flow (50 ml s^{-1}) from all flow levels (30, 100 and 300 ml s^{-1}), with F_{ENO} acquired at 50 ml s^{-1} and found a good mean agreement between the estimated and measured values. The limits of agreement between estimated $\hat{F}_{ENO}$ and F_{ENO} were reasonable.

Validation

Assessment of the conversion in external datasets, including data of a wide range of pulmonary diseases and multiple-flow F_{ENO} values, confirmed these previous findings. The conversion model developed showed the lowest deviation in F_{ENO} conversions in healthy children, healthy adults and in our

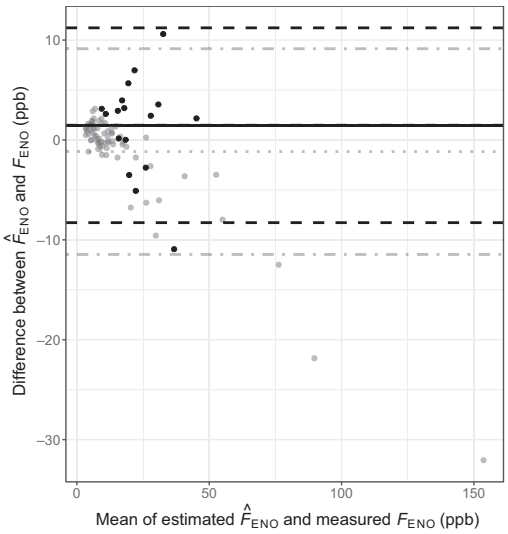

Figure 4 Bland–Altman plot with mean of measured F_{ENO} and estimated $\hat{F}_{ENO}$ from 100 ml s^{-1} in COPD patients (grey dots, n = 72) and patients with alveolitis (black dots, n = 17), plotted against the differences in F_{ENO}. In COPD patients: mean differences (grey dotted line), 1·96 standard deviations (grey dot-slashed line). In patients with alveolitis: mean differences (black solid line), 1·96 standard deviation (black slashed line). In patients with alveolitis F_{ENO} measured at 40 ml s^{-1}. In COPD patients F_{ENO} measured at 50 ml s^{-1}.

Table 2 Bland–Altman statistics with bias[a], levels of agreement and standard deviation (SD) of the differences between estimated $\hat{F}_{ENO}$ from 100 ml s^{-1} (Eq. 1) and measured F_{ENO} at 50 or 40 ml s^{-1}

		Level of agreement		
Population	**Bias[a]**	**Lower**	**Upper**	**SD**
Mixed healthy and asthmatic adults	−0·28	−7·42	6·86	3·64
Healthy adults	−0·44	−3·87	2·98	1·74
Asthmatic	−1·68	−11·36	7·99	4·94
Healthy children	0·27	−1·94	2·48	1·13
COPD	−1·16	−11·46	9·13	5·25
Alveolitis	1·47	−8·28	11·22	4·98

[a]average of the differences between estimated $\hat{F}_{ENO}$ and measured $F_{ENO\S}$.

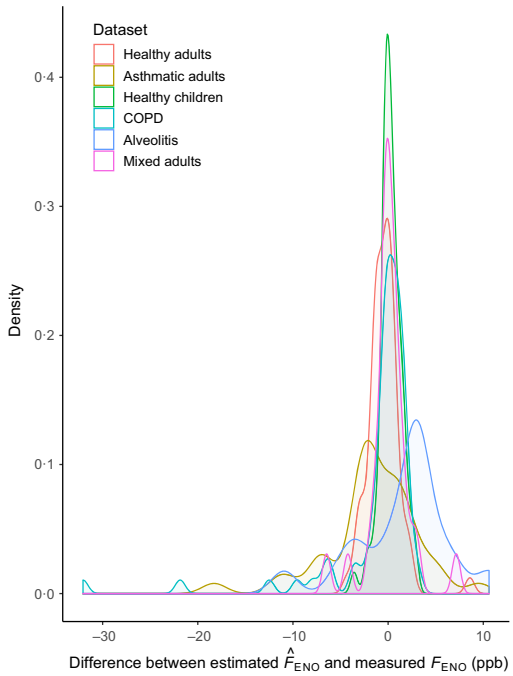

Figure 5 Density plot with mean differences between F_{ENO} measured at 50 or 40 ml s^{-1} and estimated $\hat{F}_{ENO}$ from 100 ml s^{-1}, and the density of the individual mean differences in all study groups. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Spearman's correlation between estimated $\hat{F}_{ENO}$ from 100 ml s^{-1} and measured F_{ENO} at 50 ml s^{-1}, with 95% CI and P values

		95% CI		
Population	**Correlation**	**Lower**	**Upper**	***P***
Mixed healthy and asthmatic adults	0·99	0·98	0·99	<0·001
Healthy adults	0·97	0·95	0·98	<0·001
Asthmatic	0·99	0·98	0·99	<0·001
Healthy children	0·97	0·95	0·98	<0·001
COPD	0·98	0·96	0·98	<0·001
Alveolitis	0·87	0·68	0·95	<0·001

mixed asthmatic and healthy adult population. In the steroid-naive asthmatic, alveolitis and COPD populations, the average differences in F_{ENO} were moderate with moderate limits of agreement. In the population with COPD, some single individuals showed a considerable deviation.

We acknowledge the limitation of this conversion procedure, that is being only an approximation that may result in a considerable deviation between estimated and physiological values especially at extreme F_{ENO} and/or flow levels, as observed in conversions from low flow (30 ml s^{-1}) or high expiratory flow (300 ml s^{-1}) levels. Nevertheless, this equation is useful when comparing the F_{ENO} medians of large population data measured at different flow levels, being very reliable on the group level, although not on individual level. The conversion model developed suits best F_{ENO} conversions in healthy adults, healthy children and in a mixed adult population, showing the lowest deviation. This novel conversion model mimics physiological expiratory NO values proportional to expiratory flows. Similar F_{ENO} and expiratory flow curves were previously described by other researchers

(Tsoukias & George, 1998; Silkoff *et al.*, 2000), but this model uses a simplified approach in estimating $\hat{F}_{ENO}$ and makes no claim in predicting flow-independent parameters.

Since the conversion model developed derives from healthy and asthmatic adults without alveolar diseases, the slope reflects only very low amounts of alveolar nitric oxide concentration (C_{ANO}). We previously determined C_{ANO} in our mixed healthy and asthmatic group and all results were under 2·3 ppb (Lassmann-Klee *et al.*, 2018b). Logically, the slope and the estimating equation would change, if switching the participants with subjects with high alveolar NO. The conversion method produces errors in those subjects in whom the relation between alveolar and bronchial NO production is very different from the group mean, as the slope between F_{ENO} and $\dot{V}$ is very different in these subjects. Therefore, the model may result in erroneous estimates when applied to subjects with known high alveolar nitric oxide concentrations. Emphasis should be made, not to employ the model without discretion in this type of subjects. The elimination of outliers could represent a limitation of our study, although we did not observe drastic changes when comparing the bias between crude and adjusted data. This statistical adjustment merely narrowed the limits of agreement and served the purpose of demonstrating how the model estimates F_{ENO} values stemming from adjusted datasets.

Further on, regression estimates were obtained for F_{ENO} values between the mouthwashes, in order to facilitate an interpolation between F_{ENO} values measured at 50 ml s^{-1} after carbonated, and tap water, and vice versa. Our estimating equation provides different slopes for both mouthwashes. The mean estimated $\hat{F}_{ENO}$ values were ca. 4% lower for the carbonated water mouthwash than the tap water mouthwash. This approximate difference between these mouthwashes was previously confirmed (Lassmann-Klee *et al.*, 2018a,b). The conversion model succeeds also in considering the mouthwashes.

In conclusion, we developed an equation for converting F_{ENO} values obtained with different flow levels to F_{ENO} with standard flow (50 ml s^{-1}), taking also into account the eventual mouthwash. We proposed a novel model to convert F_{ENO} in healthy populations, as well in subjects with obstructive pulmonary diseases. We conclude that the model is reliable in converting F_{ENO} in large epidemiological data and might be applied in small scale populations with pulmonary diseases, but not on individual level.

Acknowledgements

We thank the staff members: Sari Fischer, Helena Punkari and Elina Voutilainen for performing the F_{ENO} measuring and also Tommi Pallasaho for data collection.

Funding

This work was supported by the Nordic Council of Ministers, NordForsk Institution (The Nordic EpilLung Study), the Nummela Sanatorium Foundation (PP 2015, 2017), (AS 2016), Finnish State Funding for University-level Health Research (TYH: 2013354), The Research Foundation of the Pulmonary Diseases (PLK 2017, 2018, 2019), Tampere Tuberculosis Foundation: Eero Hämäläinen (PLK 2017, 2018), Ida Montin Foundation (PLK 2017, 2019), Väinö and Laina Kivi Foundation (PLK 2017, 2018, 2019), and University of Helsinki (PLK 2019).

Disclosures

No conflicts of interest are declared by the author(s).

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Respiratory Medicine 173 (2020) 106160

Contents lists available at ScienceDirect

Respiratory Medicine

journal homepage: http://www.elsevier.com/locate/rmed

Original Research

Parallel gradients in F_{ENO} and in the prevalences of asthma and atopy in adult general populations of Sweden, Finland and Estonia — A Nordic EpiLung study

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ARTICLE INFO

Keywords:
Asthma
F_{ENO}
Atopy
Skin prick test

ABSTRACT

The prevalence of asthma is higher in Sweden and Finland than in neighbouring eastern countries including Estonia. Corresponding difference in bronchial eosinophilic inflammation could be studied by F_{ENO} measurements. We aimed to compare F_{ENO} in adult general populations of Sweden, Finland, and Estonia, to test the plausibility of the west-east disparity hypothesis of allergic diseases.

We conducted clinical interviews (N = 2658) with participants randomly selected from the general populations in Sweden (Stockholm and Örebro), Finland (Helsinki), and Estonia (Narva and Saaremaa), and performed F_{ENO} (n = 1498) and skin prick tests (SPT) in 1997–2003.

The median (interquartile range) of F_{ENO} (ppb) was 15.5 (9.3) in Sweden, 15.4 (13.6) in Finland and 12.5 (9.6) in Estonia. We found the lowest median F_{ENO} values in the Estonian centres Saaremaa 13.1 (9.5) and Narva 11.8 (8.6). In the pooled population, asthma was associated with F_{ENO} ≥25 ppb, odds ratio (OR) 3.91 (95% confidence intervals: 2.29–6.32) after adjusting for SPT result, smoking, gender and study centre. A positive SPT test increased the likelihood of asthma OR 3.19 (2.02–5.11). Compared to Saaremaa, the likelihood of having asthma was higher in Helsinki OR 2.40 (1.04–6.02), Narva OR 2.45 (1.05–6.19), Örebro OR 3.38 (1.59–8.09), and Stockholm OR 5.54 (2.18–14.79).

Abbreviations: CI, 95% confidence interval; F_{ENO}, Fractional exhaled nitric oxide; FEV_1, Forced expiratory volume in 1 s; IQR, Interquartile range; OR, Odds ratio; SPT, Skin pick test.

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[1] Deceased July 2020.

https://doi.org/10.1016/j.rmed.2020.106160
Received 1 June 2020; Received in revised form 18 August 2020; Accepted 15 September 2020
Available online 17 September 2020

There was a higher prevalence of asthma and allergic airway inflammation in adult general populations of Sweden and Finland compared to those of Estonia. Atopy and elevated F_{ENO} level were independently associated with an increased risk of asthma. In conclusion, the findings support the earlier west-east disparity hypothesis of allergic diseases.

1. Introduction

Asthma is an ubiquitous chronic pulmonary disease with a high prevalence mainly in westernised countries, posing an economic burden to public health [1]. A major increase in asthma was observed during the second half of the past century [2], including Scandinavian countries such as Finland [3]. Although this rise seems to have reached a plateau in Finland during the recent years [4], the rise has apparently continued in northern [5] and western Sweden [6]. Additionally, worldwide, the prevalence of asthma still seems to be increasing [7], with hundreds of millions globally afflicted [8]. Neighbouring countries of Finland — Estonia and Russia — have been less affected with asthma [9,10]. In Estonia, a slow increase in asthma prevalence started in the last decades of the past century [11].

Early hypotheses proposed a west–east disparity in allergic diseases, including rhinitis, atopic eczema, and asthma [10,12,13]. Previous studies have shown differences in prevalence, but also in diagnostic criteria [14], as well as differences in exposure and risk factors between countries in eastern and western parts of northern Europe [15]. Researchers in Finland, Sweden and Estonia designed, in the mid-1990s, a multi-centre epidemiological study (FinEsS) to investigate allergic and obstructive respiratory diseases. A key target of the FinEsS study was to find out whether regional differences in the prevalence of asthma were reflected in regional differences of fractional exhaled nitric oxide (F_{ENO}), a biomarker of eosinophilic airway inflammation. We therefore aimed to investigate differences in F_{ENO} in general populations of Sweden (Stockholm and Örebro), Finland (Helsinki), and Estonia (Narva and Saaremaa), and the associations of F_{ENO} with asthma, asthma symptoms, smoking and skin prick test (SPT) findings to common allergens, thus assessing the west-east disparity hypothesis of allergic diseases.

2. Materials and methods

Random samples of general populations in five areas from Sweden, Finland and Estonia answered questionnaires on respiratory symptoms and diseases in 1995–1996, as part of a cross-sectional study. One to five years later, at the turn to the 21st century, random samples from these cohorts were invited to clinical examinations in all participating centres (Fig. 1), including measurements of F_{ENO}, lung function and SPT, and a structured clinical interview on symptoms of respiratory diseases.

2.1. Study populations

In 1995 and 1996, we surveyed 34 951 adults in general populations in Sweden, Finland and Estonia with a postal questionnaire (see flowchart in Fig. 2 and anthropometric and spirometric data in Table 1) sent to individuals randomised in 10-year-age cohorts from the Population Register Centres, taking into account the overall gender distribution in each local population. The postal questionnaire was answered by 27 697 (79% of invited) subjects: 6062 (76%) in Helsinki, 5754 (72%) in Stockholm, 6784 (84%) in Örebro, 4325 (78%) in Narva, and 4808 (89%) in Saaremaa. Among all these, a random sample (stratified by age and gender) of 4944 individuals (18% of those who replied to the postal questionnaires) were invited to a clinical examination. A total of 2658 (54% of invited) volunteers participated: 643 (54%) in Helsinki, 443 (37%) in Stockholm, 719 (60%) in Örebro, 402 (60%) in Narva, and 451 (67%) in Saaremaa. We further randomised these subjects and performed F_{ENO} measurements, and skin prick testing around the turn of the millennium (1997–2003) on 1669 participants. We included for this study a total of 1498 individuals with valid F_{ENO} measurements aged 20–60 years, among whom 92% had valid SPTs.

2.2. Questionnaire and clinical interview

We selected 11 questions from the clinical interview, including those questions with reference to self-reported or diagnosed obstructive respiratory diseases, asthma symptoms, asthma medication, smoking, and self-reported allergic rhinitis or conjunctivitis. The postal questionnaire was based on the OLIN (Obstructive Lung Diseases in Northern Sweden) research project and further on the British Medical Research Council and Tucson questionnaires [3]. The clinical interview was based on the former postal questionnaire, but augmented with detailed questions of symptoms, medication, and possible risk factors for obstructive airway diseases and allergy. Translations of the questionnaire were available in all native languages (Finnish, Swedish, Estonian, and Russian). Independent translations of the questionnaires from Swedish to other languages were produced by bilingual translators who were aware of the objective of the study and had expertise in the study topic.

Fig. 1. Map of the FinEsS research centres in Sweden, Finland and Estonia. Map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under ODbL.

2.3. Definitions

The definitions presented below were based on **affirmative answers** to each question.

Asthma: have you ever had asthma?

Asthma diagnosis: Have you been diagnosed with asthma by a physician?

Childhood asthma: Have you had asthma during childhood or had a wheeze during breathing in early childhood?

Chronic obstructive pulmonary disease (COPD) *diagnosis*: Have you been diagnosed with chronic bronchitis or emphysema by a physician?

Current smoker: are you a current smoker?

Allergic rhinitis or conjunctivitis (ARC): Have you or have you had hay fever (allergic rhinitis) or allergic eye inflammation?

Asthma symptoms during the last year: Have you had any asthma symptoms during the last 12 months?

Nightly asthma symptoms last year: Have you ever been woken up during the night or early morning by an attack of shortness of breath with wheezing? If yes, has this happened during the last 12 months?

Asthma medication during the last year: Have you used any asthma medicines during the last 12 months?

Inhaled corticosteroids (ICS) *last year*: Have you used inhaled corticosteroids during the last 12 months (List of national commercial names)?

Short acting β-agonists (SABA) *last year*: Have you used inhaled short acting β-agonists during the last 12 months (List of national commercial names)?

2.4. F_{ENO} measurements

F_{ENO} was measured according to the European Respiratory Society (ERS) guidelines [17], but the expiratory flow rate varied between the study centres. All participating centres employed a chemiluminescence nitric oxide (NO) analyser, Sievers 270B (Boulder, CO, USA). The devices were calibrated daily (two-point calibration). The gas used for inhalation was NO-free synthetic air. The exhalation was visually controlled and flow resistors were used (Hans Rudolph Inc., Shawnee, KS, USA) in order to control the target flow range and to close the soft palate. The expiratory flow rates were: 250 mL/s in Stockholm and Örebro, 50 mL/s in Helsinki, and 100 mL/s in Narva and Saaremaa. The exhalation time was at least 6 s. The mean F_{ENO} obtained for analysis comprised a mean value of three consecutive end-of-exhalation plateau determinations. No nose-clips were used for the manoeuvres, but mouthwashes were performed prior to the measurements: in Helsinki with carbonated water, and in other centres with tap water, in order to reduce oral NO contamination [18,19].

Since the study centres utilised different expiratory flows in F_{ENO} measurements, all F_{ENO} values had to be converted to values with a 50 mL/s flow, the recommended value by the American Thoracic Society (ATS) and ERS [20], for comparison between the study centres. We used a conversion model developed by Lassmann-Klee et al. [21], which accounts for different flow-rates and different mouthwashes.

We selected a F_{ENO} value of $\geq$25 ppb as an intermediate cut-off value according to ATS and ERS [22,23].

2.5. Skin prick tests

We tested 92% of the participants with SPTs for the following allergens: dog, cat, horse, timothy-grass, birch, mugwort, moulds (*Alternaria alternata*, *Cladosporium herbarum*), and house dust mites (*Dermatophagoides pteronyssinus, Dermatophagoides farinae)*. The testing protocol was previously described elsewhere [24]. All centres, apart from Stockholm, arranged additional testing for storage mites (*Lepidoglyphus destructor*, *Acarus siro*), cow, cockroach (*Blatella germanica*), and latex. We defined a volunteer as atopic, if at least one positive SPT was observed, i.e. a skin reaction of $\geq$3 mm, after rejecting cases with dermographism [25].

2.6. Spirometry

We used different spirometers in the participating centres: Ohio spirometer (Stockholm), Volugraph 2000 and Vitalograph (Örebro), SensorMedix Vmax22 (Helsinki) and Mijnhardt Vicatest 5 (Narva and Saaremaa). We acquired forced expiratory volume in 1 s (FEV_1) only (Table 1). The highest value of three acceptable FEV_1 values was recorded.

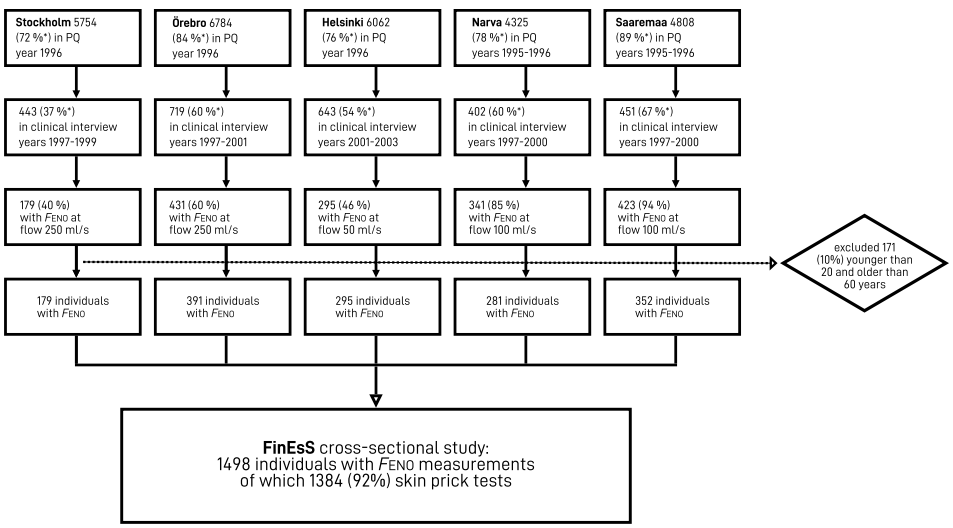

Fig. 2. Flowchart of population selection in Finland, Sweden and Estonia (FinEsS). Presented as *n*, (%*) percentage of invited, or (%) percentage of total. Postal questionnaire (PQ), fractional exhaled nitric oxide (F_{ENO}).

2.7. Statistics

All statistical tests were performed in *R* [26]. Due to a non-normal distribution of F_{ENO} in the general population, we tested the differences between the centres and countries with a Kruskal–Wallis test, with a post-hoc Dunn's test for multiple comparison and Holm's adjustment.

F_{ENO} distributions in two-group variables were compared with a Wilcoxon–Mann–Whitney–Test for independent groups.

We calculated raw odds ratios (OR) with a two-by-two table, the 95% confidence intervals (CI) using the method number ten by Newcombe [27] and corresponding *p* values with Pearson's χ^2 test with Yates' continuity correction. The adjusted ORs were calculated by binary logistic regression. Within the binary logistic regression, we tested the multiple parameters of the study centre variable with Wald χ^2 statistics. The best fitting models were selected by using the Akaike information criterion (AIC).

We calculated a minimum sample size of $n = 1232$ for a binary logistic regression when considering an odds ratio of 1.5 for an event and a prevalence of 4%, according to Hsieh et al. [28].

2.8. Ethics

The study was approved by the Ethics Committee of Department of Medicine of Helsinki University Hospital, the Tallinn Ethics Committee in Estonia and the Swedish Ethical Review Authority. All individuals filled in a written informed consent form attached to the structured interview.

3. Results

Totally, 1498 persons were included in the analyses from five centres. Age, height, weight, BMI, and FEV_1 results were similar in the study centres (Table 1).

3.1. Prevalence of symptoms and diagnoses

The diagnoses, respiratory symptoms, and median F_{ENO} varied significantly between the areas (Table 2). The population in Stockholm had a high prevalence of asthma (13%), asthma diagnosis (11%), asthma symptoms last year (13%) and asthma drug use (ICS 9%, SABA 10%), while the population in Saaremaa had the lowest prevalences of asthma (3%), asthma diagnosis (2%), asthma symptoms, and asthma drug use. We found the highest prevalence of COPD diagnosis (18%) and current smokers (41%) in the population of Narva. The studied sample from Helsinki had the highest prevalence of allergic rhinitis or conjunctivitis (ARC) (40%) and atopy (49%).

3.2. Fractional exhaled nitric oxide: F_{ENO}

The median (interquartile range (IQR)) F_{ENO} was 14.0 (10.5) ppb in the pooled sample, and 15.5 (9.3) in Sweden, 15.4 (13.6) in Finland and 12.5 (9.6) in Estonia (Kruskal–Wallis test, $p < 0.001$). The post-hoc analysis showed differences in median F_{ENO} between Estonia and the other countries ($p < 0.001$). Median F_{ENO} varied significantly between the centres ($p < 0.001$) (Fig. 3). We found lower median F_{ENO} ($p < 0.001$) values in Estonian centres, with differences between Narva and Helsinki ($p < 0.001$), Narva and Stockholm ($p = 0.001$), and Narva and Örebro ($p < 0.001$); and as well as significant differences between Saaremaa and Helsinki ($p = 0.002$), and Saaremaa and Örebro ($p < 0.001$).

3.3. Between country comparisons for asthma

The likelihood of having asthma was higher in Sweden and Finland than in Estonia, with crude *OR 2.39(1.48-3.85)* and *2.0(1.12-3.58)*, respectively. Comparing Sweden with Finland, we found similar likelihood of having asthma, crude *OR 1.19(0.71-2.0)*.

3.4. F_{ENO} and asthma

We found that self-reported asthma was associated with F_{ENO} ≥25 ppb, *OR 3.91(2.39-6.32)*, after adjusting for SPT result, smoking, gender and study centre in a binary logistic regression model (Fig. 4) (Model can be found in the Appendix). Within this same model, we found that the overall effect of the study centre in general ($p < 0.01$) was a significant factor associated with asthma. Analysing the differences between the study centres within the model, we found that compared to Saaremaa, the likelihood of having asthma was higher in Helsinki *OR 2.40(1.04-6.02)*, Narva *OR 2.45(1.05-6.19)*, Örebro *OR 3.38(1.59-8.09)*, and Stockholm *OR 5.54(2.18-14.79)*. Within the model, asthma was associated with a positive SPT result, *OR 3.19(2.02-5.11)*, but not with smoking *OR 0.87(0.50-1.46)* or gender *OR 0.81(0.51-1.27)*, in the whole population.

In the total pooled sample, the mean F_{ENO} was higher in participants with asthma (27 ppb) than without asthma (16 ppb) ($p < 0.001$), see Table 3. When stratifying for current smoking status, the mean F_{ENO} was higher in non-smokers, than in smokers ($p < 0.01$), both in asthmatics and in non-asthmatics. Further stratification revealed a higher mean F_{ENO} in atopic asthmatic individuals, than in non-asthmatics ($p < 0.001$) with atopy. Analogously, non-atopic asthmatic individuals had a higher mean F_{ENO} compared to non-asthmatics ($p = 0.016$) without atopy.

3.5. F_{ENO} ≥25 ppb

In the pooled sample, individuals with F_{ENO} ≥25 ppb were more likely to have atopy, self-reported asthma, physician diagnosed asthma, and allergic rhinitis (Table 4). Participants with F_{ENO} ≥25 ppb were more likely to report asthma symptoms, nightly symptoms, ICS use, and SABA use, in the previous year. Current smokers were more likely to have a F_{ENO} result <25 ppb. A result of F_{ENO} ≥25 ppb was not associated with COPD diagnosis or childhood asthma.

The prevalence of F_{ENO} ≥25 ppb in the pooled sample was 14.6%, with 14.7% in Sweden, 21.4% in Finland, and 11.2% in Estonia. In the centres, the prevalence of F_{ENO} ≥25 ppb was 10.6% in Stockholm, 16.6% in Örebro, 21.4% in Helsinki, 11.4% in Narva, and 11.1% in Saaremaa.

The ORs (95% CIs) for F_{ENO} ≥25 ppb, compared to Finland, were

Table 1
Anthropometric data and forced expiratory volume in 1 s (FEV1) in adult general populations of Stockholm, Örebro, Helsinki, Narva, and Saaremaa.

		Total	Stockholm	Örebro	Helsinki	Narva	Saaremaa
	n	1498	179	391	295	281	352
Gender	f/m	824/674	99/80	199/192	174/121	162/119	190/162
	%	55/45	55/45	51/49	59/41	58/42	54/46
Age (years)	mean (sd)	40.9 (10.9)	39.3 (10.0)	42.1 (10.8)	40.9 (10.2)	40.2 (11.6)	41.0 (11.4)
Height (cm)	mean (sd)	171.1 (9.3)	172.9 (9.6)	172.2 (9.2)	170.0 (9.3)	168.6 (8.7)	171.7 (9.3)
Weight (kg)	mean (sd)	75.3 (14.7)	72.7 (13.5)	75.5 (13.8)	75.2 (16.2)	73.7 (13.5)	77.6 (15.7)
BMI(kg/m^2)	mean (sd)	25.7 (4.5)	24.2 (3.5)	25.4 (3.9)	26.0 (5.0)	25.9 (4.4)	26.3 (5.0)
FEV_1 (L)	mean (sd)	3.4 (0.9)	3.7 (0.8)	3.2 (0.8)	3.3 (0.9)	3.4 (0.9)	3.6 (0.9)

f = female, m = male, sd = standard deviation.

Table 2
Median F_{ENO} (interquartile range (IQR)) and prevalence of asthma, respiratory symptoms within the last year, and atopy in Stockholm, Örebro, Helsinki, Narva, and Saaremaa.

		Stockholm	Örebro	Helsinki	Narva	Saaremaa	p
F_{ENO} median (IQR)		14.4 (8.0)	**15.7(10.1)**	15.4 (13.6)	11.8 (8.6)	13.1 (9.5)	<0.001†
		%	%	%	%	%	
Ever asthma	Yes	**12.8**	8.5	9.1	6.3	2.9	<0.001
Asthma by MD	Yes	**11.4**	7.8	7.2	5.4	2.3	<0.001
Asthma in childhood	Yes	8	**8.6**	7.3	1.8	6.4	<0.01
Asthma symptoms	Yes	**13.4**	9.2	12.5	6.1	2.6	<0.001
Nightly symptoms	Yes	**7.3**	4.9	1	6.4	4.3	<0.01
Asthma medication	Yes	**12.3**	6.9	9.2	4.6	2.3	<0.001
ICS used	Yes	**8.9**	4.1	5.1	1.1	0.6	<0.001
Saba used	Yes	**10.1**	5.9	4.4	3.6	2.3	<0.01
Atopy	Positive	21.2	33.5	**48.5**	37.7	25.8	<0.001
Allergic rhinitis	Yes	33	39.4	**40**	32.4	17.6	<0.001
Current smoking	Yes	37.2	22	37	**40.5**	34.1	<0.001
COPD by MD	Yes	2.9	1.31	2.8	**17.8**	9.2	<0.001

MD = medical doctor, COPD = chronic obstructive pulmonary disease, ICS = inhaled corticosteroid, SABA = short-acting β_2-agonist, bolded numbers represent the highest prevalence †p values calculated with Kruskal-Wallis test for the differences in $F_{ENO,}$ for the others Pearson's χ^2 test for the interviews' answers and atopy (skin prick tests).

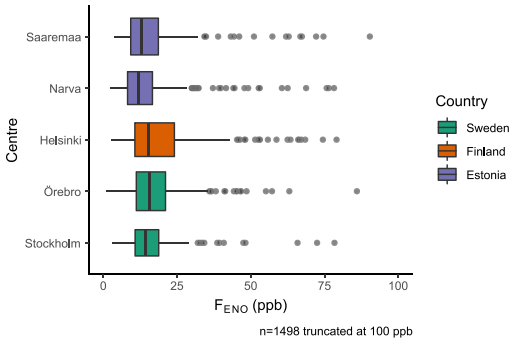

Fig. 3. Boxplot with fractional exhaled nitric oxide (F_{ENO}) values of general adult populations from the participating centres: Helsinki ($n = 295$), Stockholm ($n = 179$), Örebro ($n = 391$), Narva ($n = 281$), and Saaremaa ($n = 352$) grouped by countries.

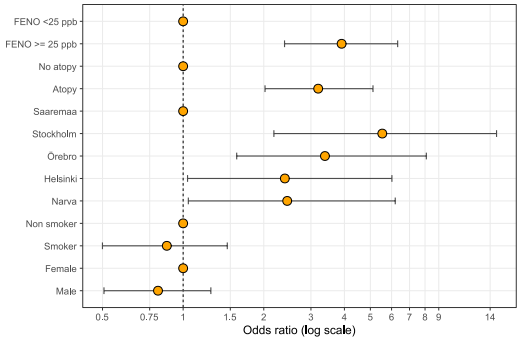

Fig. 4. Binary logistic regression model with asthma as outcome and $F_{ENO} \geq 25$ ppb, participating centre, gender, smoking, and atopy as predictors. Presented as odds ratio (OR) with 95% confidence intervals. Reference groups: F_{ENO} <25 ppb, no atopy, Saaremaa, non-smoker and female.

0.60 (0.41–0.89) in Sweden and 0.48 (0.33–0.72) in Estonia, and adjusted for positive SPT 1.52 (1.11–2.07), smoking 0.33 (0.22–0.49), and male gender 1.36 (1–1.85). Similarly, compared to Helsinki, the adjusted OR for $F_{ENO} \geq 25$ ppb was 0.42 (0.21–0.81) in Stockholm, 0.65 (0.43–0.98) in Örebro, 0.53 (0.32–0.84) in Narva and 0.45 (0.28–0.71) in Saaremaa.

4. Discussion

We observed a west–east gradient of F_{ENO} levels and prevalences of asthma and asthma symptoms in the studied populations from Sweden, Finland and Estonia. In the pooled samples, we found a higher median F_{ENO} in Sweden and Finland compared to Estonia. The Estonian centres had a lower median F_{ENO} compared to Swedish and Finnish centres. The Estonian populations and the pooled Estonian sample also had lower adjusted ORs for asthma.

Previous investigations on these Nordic populations hypothesised diagnostic differences and labelling of asthma as potential explanations for the lower prevalence of asthma and its symptoms in Estonia [14], as well as possible low physician diagnosis rates [11]. Nevertheless, we found significantly different F_{ENO} levels in the regions studied, supporting the view, that there is a real disparity in asthma prevalences between the studied centres, reflecting differences in allergic airway diseases. The reasons for the west–east gradient in F_{ENO} and asthma prevalence may be based, for example, on differences in genetic, geographical, environmental, occupational and socio-economical risk factors and lifestyle, and on protective factors, like rural living. A previous comparison between Uppsala in Sweden and Tartu in Estonia found a lower prevalence of atopy, and a lower prevalence of pollen-associated asthma symptoms in Estonia than in Sweden [29]. However, a previous FinEsS study found, apart from similar prevalence of sensitisation in Sweden, Finland and Estonia, a deviant sensitisation pattern: storage mites and cockroach were major sensitisers in Estonia [30], while sensitisation in Finland and Sweden was mainly attributable to pollen and furry animals [24,31]. Moreover, marked differences in the sensitisation levels have been found between North Karelia in Finland and the Republic of Karelia in Russia, with higher asthma prevalence in Finnish Karelia [32]. Remarkably, another study in Uppsala and Tartu found lower mean eosinophilic cationic protein (ECP) blood levels, a biomarker of eosinophilic inflammation, in Tartu [33], further endorsing the findings of the present study. Farm living was previously found to reduce the risk for allergy and asthma development [6,34,[35]], and this could partly explain the lower asthma risk in Saaremaa, an Estonian island with a high degree of rurality. On the other hand, Narva is a highly industrialised and air-polluted area next to the

Table 3
Fractional exhaled nitric oxide (F_{ENO}) mean (SD) stratified by smoking and atopic status in participants with or without asthma.

		Asthmatics (n = 103)				Non asthmatics (n = 1329)			
F_{ENO} (ppb)	mean (sd)	27 (29)				16.3 (11)			
		current non smoker		current smoker		current non smoker		current smoker	
	n (%)	80 (73%)		23 (22%)		903 (68%)		422 (32%)	
F_{ENO} (ppb)	mean (sd)	29.8 (31.6)		17.1 (12.9)		18 (11.6)		12.8 (8.8)	
F_{ENO} ≥25 ppb	%	32 (40%)		4 (17%)		142 (16%)		26 (6%)	
		atopic	non-atopic	atopic	non-atopic	atopic	non-atopic	atopic	non-atopic
		Σn = 67		Σn = 21		Σn = 841		Σn = 393	
	n (%)	42 (63%)	25 (37%)	13 (62%)	8 (38%)	271 (32%)	570 (68%)	130 (33%)	263 (67%)
F_{ENO} (ppb)	mean (sd)	31.5 (37.3)	30.9 (22.5)	21.7 (14.9)	11.3 (7.1)	19.9 (14.2)	17 (9.8)	13.3 (11.4)	12.4 (7.4)
F_{ENO} ≥25 ppb	n (%)	17 (41%)	13 (52%)	3 (10%)	1 (13%)	53 (20%)	80 (14%)	10 (7.7%)	15 (6%)

Data shown as n (%) or mean (standard deviation).

Table 4
Univariate crude odds ratio (with 95% confidence intervals) in the pooled sample for F_{ENO} ≥25 ppb and diagnosis, respiratory symptoms and medication within last year, and current smoking.

		Odds ratio	Confidence Intervals	p
Ever asthma	Yes	3.71	2.40–5.74	<0.001
Asthma by MD	Yes	2.78	1.74–4.44	<0.001
Asthma in childhood	Yes	1.35	0.78–2.33	0.358
Asthma symptoms	Yes	3.1	2.06–4.68	<0.001
Nightly symptoms	Yes	2.04	1.15–3.60	0.02
Asthma medication	Yes	3.22	2.05–5.05	<0.001
ICS used	Yes	2.48	1.34–4.60	0.005
Saba used	Yes	3.40	2.04–5.65	<0.001
Atopy	Positive	1.62	1.20–2.19	0.002
Allergic rhinitis	Yes	2.11	1.58–2.83	<0.001
COPD by MD	Yes	1.09	0.61–1.92	0.897
Current smoking	Yes	0.33	0.23–0.49	<0.001

MD = medical doctor, COPD = chronic obstructive pulmonary disease, ICS = inhaled corticosteroid, SABA = short-acting β_2-agonist.

Russian border, with an ethnic Russian majority [9], and high prevalences of smoking (40.5%) and COPD (17.8%). A recent study analysing the air concentrations of fine particles, benzene, and phenol, showed a similar prevalence of self-reported asthma in Narva, as in our present study [36]. The environmental factors, smoking and occupational conditions could partly explain the low median F_{ENO}, since our analyses revealed a slightly higher likelihood of asthma in Narva compared to Saaremaa.

Previous studies observed a decrease of F_{ENO} levels by current smoking [37–39], and others have also suggested a similar effect by past smoking [39,40]. An experimental study in lung epithelial cells discovered a possible mechanism for the effect of cigarette smoke on F_{ENO} production, down-regulation of an inducible nitric oxide synthase enzyme (iNOS) [41]. Our stratified analyses corroborate the lower F_{ENO} levels in smokers, independently of asthma or atopy.

As expected, a F_{ENO} level ≥25 ppb was strongly associated with asthma in the pooled population. Likewise, a positive SPT result was associated with asthma. A previous study from an Icelandic population, in which F_{ENO} ≥25 ppb was positively associated with asthma and a positive SPT, found a similar prevalence of F_{ENO} ≥25 ppb as in our Finnish population [42].

Previously, atopy and smoking were found to be associated with F_{ENO} in Scandinavian populations [42–44]. Nevertheless, a previous investigation in Helsinki could not find differences of F_{ENO} between SPT negative and SPT positive healthy asymptomatic non-smokers [45]. Thus, elevated F_{ENO} in atopic subjects indicates an allergic airway disorder. In concordance with a large study of F_{ENO} in Scandinavia [44], we showed here that atopic subjects frequently have elevated F_{ENO} levels regardless of smoking.

This is, as far as we know, the largest multi-centre study on F_{ENO} in populations of Northern European countries, including skin prick testing and a structured interview. The study reflects the era after the Estonian Restoration of Independence and the end of the Soviet occupation during the 1990's, which apart from its historical importance, marks a change in lifestyle, occupational and socio-economical conditions. At the same time, Finland and Sweden were already immersed in a westernised lifestyle with a higher socio-economical status. This background gives unique possibilities to study the differences in the prevalences of asthma and atopy and to find out differences in F_{ENO} levels. Previously, an increase in atopic sensitisation was found after the German Reunification in schoolchildren in former East Germany during the 1990's [2].

One strength of the study is, that self-reported parameters were compared with measured F_{ENO} and skin-prick test data. Weaknesses of the present study could be, apart from employing self-reported parameters, a possible selection bias through multiple inclusion steps and the differences in expiratory flows in the original F_{ENO} measurements. Nevertheless, we employed similar F_{ENO} devices and applied a validated conversion model to standardise F_{ENO} to the recommended expiratory flow of 50 mL/s [21] to obtain comparable values. The standardised F_{ENO} values are similar to those obtained in large general populations studies [46] and also by applying a reference equation to our pooled population [47]. The F_{ENO} value obtained in Örebro in the present study is in harmony with the values found in a general population study from Gothenburg in Sweden [44], which employed the recommended expiratory flow of 50 mL/s.

In conclusion, the median F_{ENO} was lower in the general adult populations in Estonia, than in Sweden or Finland. We found that F_{ENO} followed a west-east gradient, parallel with the differences in asthma prevalence in the studied centres and a higher likelihood of asthma in western populations. The findings support the earlier west-east disparity hypothesis of allergic diseases. We also showed here that atopy and increased F_{ENO} values (≥25 ppb) were independently positively associated with asthma, confirming earlier studies.

Author contributions

BL and AS conceived and designed the research. ML, MM, AS, and B-MS supervised the experiments. PLK analysed the data and wrote the manuscript, under the tutelage of PLP and AS. All authors read and approved the manuscript.

Acknowledgments

We thank staff members from the Skin and Allergy Hospital and the Laboratory of Clinical Physiology at the Meilahti Hospital, and Alyce Whipp, MPH for the language revision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rmed.2020.106160.

Funding

This work was supported by: Foundation of the Finnish Anti-Tuberculosis Association, Finland; The Research Foundation of the Pulmonary Diseases, Finland; Tampere Tuberculosis Foundation: Eero Hämäläinen, Finland; Väinö and Laina Kivi Foundation, Finland; Ida Montin Foundation, Finland; Estonian Science Foundation, Estonia; Nummela Sanatorium Foundation, Finland; the University of Helsinki, Finland; and the Nordic Council of Ministers: NordForsk (Nordic Epi-Lung Study), Norway.

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