Exhaled nitric oxide in infants is related to housing characteristics

Report of scientific project for University Medical Centre Utrecht
Transition Year CRU ’99

Makowska DE, Carlstedt F, Hasselgren M

Exhaled nitric oxide (eNO) is a non-invasive marker of airway inflammation. This study investigated the methodological aspects of eNO measurement with offline tidal breathing technique in infants compared to the on-line single breath method in their mothers and their housing characteristics. The participation rate was 42.5%, eNO was measured in 53 children aged two and six months and in 52 mothers. The median eNO level was similar for infants with 13.1 parts per billion (ppb) and their mothers, 12 ppb. There was, however, no sign of correlation between their eNO values. Median eNO was significantly higher in children living in the centre of the city than living in the rural area, 19.2 versus 8.5 ppb. eNO in infants living in large homes was significantly lower than in ones living in small homes, 10.5 and 18.9 ppb respectively. Healthy mothers had significantly lower eNO values compared to mothers with self-reported asthma and/or allergic rhinitis; 10.0 and 16.0 ppb respectively.

These results indicate that eNO measured by offline tidal breathing technique in infants can be used in a longitudinal study as a marker of inflammation and eNO was found to be related to some building characteristics or living conditions.

Asthma and allergic diseases are common and important public health problems with a growing prevalence and still not fully understood etiology. Allergic diseases usually start in early childhood with food allergy and atopic dermatitis, followed by asthma and rhinitis, the so called allergic march.

The prevalence of allergic disease has increased several folds since the 1960, especially in children. This relatively short time-span makes genetic changes unlikely. Therefore the causes must be sought in the environment. Foetal environment and early life factors are suggested to programme risk of allergic disease in later life (1).

There is a scientific debate whether the main causes are to be sought in a changing human infectious panorama, the hygiene hypothesis, or alternatively in a changing exposure to new chemical compounds. The Swedish cross-sectional study Dampness in Buildings and Health showed that asthma and allergies among children are associated with chemical exposures such as phthalates from plasticized PVC, organic compounds associated with cleaning products and a low ventilation rate in the house (2).

As cross-sectional studies only can suggest associations, it is necessary to have prospective cohort studies with a mother-child design in order to better understand the contributing environmental factors. One such study is the Generation R study in Erasmus Medical Centre Rotterdam, the Netherlands (3). In this study a novel method to measure inflammation in preschool children is used: the assessment of exhaled nitric oxide (eNO).

Nitric oxide (NO) is an important endogenous biological messenger regulating vascular tonus and taking part in the immune response. In 1993 it was found that eNO is elevated in patients with asthma (4). Several studies have evaluated the role of eNO in inflammatory airway disorders, in particular asthma in adults and in school-age children (5–7).

There are a number of techniques to measure eNO; single breath and tidal breathing measurements. Both can be performed online - directly analyzed, or offline - gas collected in a reservoir for later analysis. The single-breath exhalation, the recommended technique for adults and school aged children (8), requires breathing manoeuvre with a constant flow and measures the last portion of exhaled air, from

Keywords: infants, nitric oxide off line tidal breathing measurement, housing
the lower airways. This method is not applicable in young children, who cannot actively cooperate. However, an offline tidal breathing technique with uncontrolled flow is potentially useful in infants and young children.

There are several factors that influence the level of eNO. One is that NO is produced by nasal mucosa and paranasal sinuses (9). A face mask with septation between oral and nasal cavity or a face mask placed only over the mouth can be used in order to avoid the nasal contamination, but these procedures can cause distress in infants since they mostly nose breath (10). Another factor is atmospheric ambient NO, which can fluctuate from 2 to 300 parts per billion (ppb), depending on traffic emissions or season of the year (11, 12). If ambient NO is more than 5 ppb, it is recommended to inhale the NO-free air prior to eNO measurement, a procedure that also might disturb the youngest children. In addition intake of nitrate-rich products as lettuce or spinach transiently elevates eNO (13, 14). Breastfeeding before measurement in infants does not influence eNO values (10). A more common cause of raised eNO is airways infections, which may elevate eNo for a week afterwards (10, 12).

Swedish Environmental Longitudinal Mother and child study on Asthma and allergy (SELMA) is a planned prospective study, see www.selmastudien.se. The aim of SELMA is to examine the relation between the indoor environment and the development of allergic disease. Data will be collected by written questionnaires, collecting of dust from the participants’ homes, sampling of blood and urine in pregnant women and their offspring. SELMA is planned to be incorporated into the ordinary health care screening programme. Measurements will be done during the regular visits of pregnant women by the midwife and later in children by physicians and nurses in the Primary Health care centres.

In prospective studies the endpoints are usually manifest disease, but it would be valuable to have an early and non-invasive marker of airway inflammation at hand prior to manifest disease. Such markers could be NO, or urinary eosinophilic protein X (EPX) and leukotrien E4 (LTE4).

This study, called NO In Longitudinal Studies (NILS) is a pilot project before SELMA starts. It aims to investigate the methodological aspects of eNO sampling using the offline tidal breathing technique with uncontrolled flow rate in infants and comparing this to on-line NO measurement in the children’s mothers and their living environment. A secondary aim was to evaluate if eNO and EPX or LTE4 in urine correlate to each other or to housing characteristics of the participants, those results are not given in this paper.

**Material and methods**

The project was approved by the local ethics committee and informed consent was obtained from all participants prior to inclusion. This study had an estimation of power in the ethical application, to show significant differences required at least 100 mother-child pairs. The study estimated a participation rate of 60-80%, requiring about 150 possible mother-child pair.

**Subjects**

The study took place from March to August 2007 in four Primary Health Care Centres (PHCC): Kronoparken, Gripen, Rud and Filipstad in Värmland, Sweden. Värmland is a county on the border to Norway, nearly half the size of the Netherlands with a population of 313.000 inhabitants and 30 PHCC. Consecutive infants with mothers who were scheduled for a regular two or six months visit to a physician were invited to participate by an information letter sent to the parents. The letter included description of the study, an informed consent, instruction concerning urine sampling and a written questionnaire.

**Methods**

Parents consenting to take part in the study handed in the urinary samples and questionnaire. All children underwent standard examination, prior to the eNO-measurements. The measurement was done before a planned vaccination.
On the day of examination ambient NO level in the air was measured. Exhaled NO in infants was measured offline after tidal breathing through a facemask (Hans Rudolph Inc). The infants should be awake and calm. The unseparated facemask was placed over the mouth and the nose. Five ordinary breaths of mixed oral and nasal expired air were collected into the gas sampling Mylar balloon via a non-re-breathing valve, figure 1–3. The balloons were of Dutch origin, from a manufacturer of helium balloons. The NO concentration in the exhaled mixed oro-nasal air was measured offline within 24 hours with the CLD 77 AM nitric oxide chemiluminescence analyzer (ECO Physics GmbH, Dürnten, Switzerland) which was calibrated prior to measurements. The analyzing of breath samples took place in the Primary Care research Unit in Kronoparken, Karlstad. Exhaled NO concentration in mothers was measured online with the handheld machine NIOX MINO® (Aerocrine, Stockholm, Sweden). This analyzer measures eNO during constant flow against resistance to obtain closure of the soft palatine. The procedure is shown in figure 4 (15).

Urine sampling

Two samples of morning urine from mothers and two samples from children were obtained according to the instruction included in the letter sent to the participants. Urine from children has been collected by using a sanitary towel, a form of menstrual protection, made of cellulose tissue put in the regular diaper on the night before coming to the PHCC. Using included plastic gloves the sanitary towel was squeezed out for urine and pored into two laboratory tubes. All tubes were sent to the laboratory, registered and frozen in a bio-bank.

Written Questionnaire

The families filled in a questionnaire with 33 multiple choice questions. The questions had previously been used in the DBH study and concerned allergy or other disease in the family, smoking habits, pets, size, type and location of housing, humidity problems in the houses and type of material in flooring in the bedrooms of the child and the mother (2). Upon examination information on recent upper airway infection and large recent intake of nitrate rich food was filled in the questionnaire. The weight and length of the child was recorded and asked for in mothers.
1. Empty lungs

2. Inhale deeply through disposable filter.

3. Exhale through filter.

4. View result on the NIOX MINO display.

Figure 4. Procedure to measure fractioned exhaled NO with NIOX MINO®. The participant can see the display of NioxMino in the mirror, which simplifies the breathing manoeuvre.

Statistical analysis
Statistical analysis of collected data was performed with SPSS 14.0. Descriptive data that was normally distributed is given with mean and standard deviation. For non-normal distributions non-parametric Chi-square test was used for proportions and Mann-Whitney U test for differences between groups. For paired data the Wilcoxon test was used. A p-value below 0.05 was considered to be statistically significant.

Results
Letters were sent to 146 mothers and their children and 62 accepted to participate. Of these 62, 9 were excluded because of different reasons, mainly technical problems with the offline NO-analyzer, see figure 5. The technical problems, with the analyzer on repair for several weeks, hampered recruitment of mother-children during the study period. Eventually 53 infants and 52 mothers, one mother of twins, were included. Demographic characteristics of the study population are presented in the tables 1–3. There were more girls than boys in the included group, but the age distribution was equal.

Figure 5. Recruitment flow chart
Table 1. Difference of proportion, Chi-square test between included and non-participants in 146 infants invited to participate

<table>
<thead>
<tr>
<th></th>
<th>Included infants</th>
<th>Non-participants</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>boys</td>
<td>23 (31%)</td>
<td>51 (69%)</td>
<td>0.048</td>
</tr>
<tr>
<td>girls</td>
<td>30 (48%)</td>
<td>33 (52%)</td>
<td></td>
</tr>
<tr>
<td>2 months old</td>
<td>25 (39%)</td>
<td>39 (61%)</td>
<td>0.933</td>
</tr>
<tr>
<td>6 months old</td>
<td>28 (38%)</td>
<td>45 (62%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Demographic characteristics of included infants with mean value and standard deviation (SD), n=53

<table>
<thead>
<tr>
<th>Infants</th>
<th>Length in cm mean (SD)</th>
<th>Weight in grams Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 months (n=25)</td>
<td>58.2 (2.2)</td>
<td>5502 (681)</td>
</tr>
<tr>
<td>6 months (n=28)</td>
<td>66.6 (2.1)</td>
<td>7929 (1182)</td>
</tr>
</tbody>
</table>

Table 3. Age in years and body mass index (BMI) of included mothers with mean value and standard deviation (SD)

<table>
<thead>
<tr>
<th>Mothers</th>
<th>Age Mean (SD)</th>
<th>BMI* Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=52</td>
<td>32 (4.6)</td>
<td>24.8 (4.5)</td>
</tr>
</tbody>
</table>

*BMI weight in kg/length in meters x length in meters.

**eNO in infants**

All included infants were healthy at the time of measurements. None of them had a chronic disease. Six of them had an upper airway infection in the two weeks prior to the examination. No mothers had eaten large amounts of spinach or salad at the measurement’s day. Eight of them had a chronic disease: five mothers reported asthma of which three had inhaled corticosteroids; three women had respectively diabetes mellitus, hypothyroid or irritable bowel syndrome. No mother had acute symptoms on measurements. Six reported upper airway infection in the two weeks prior to the measurement.

Table 4. Median and interquartal range of eNO in parts per billion (ppb)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>eNO (1-3 quartile )</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mothers</td>
<td>52</td>
<td>12.0 (8.0–17.0)</td>
<td>0.64</td>
</tr>
<tr>
<td>children</td>
<td>53</td>
<td>13.1 (8.2–18.8)</td>
<td></td>
</tr>
<tr>
<td>boys</td>
<td>23</td>
<td>13.1 (8.2–17.2)</td>
<td>0.71</td>
</tr>
<tr>
<td>girls</td>
<td>30</td>
<td>13.8 (7.8–19.5)</td>
<td></td>
</tr>
<tr>
<td>2 months old</td>
<td>25</td>
<td>12.5 (6.8–20.0)</td>
<td></td>
</tr>
<tr>
<td>6 months old</td>
<td>28</td>
<td>14.8 (9.4–18.7)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

\(a\) Wilcoxen test of pared data  
\(b\) Mann-Whitney U test

On all measurements days the ambient NO was less than 5 ppb. All participating infants were awake and breathing calmly during the measurements. Only one excluded child cried and could not participate.

The eNO values were non-normally distributed and therefore medians and interquartal range are given in table 4. Infants living in the centre of the city, \(n=24\) had significantly higher eNO levels than children living in the rural areas \(n=8\), median 19.2 and 8.5 respectively, \(p=0.005\), figure 6.
The size of the homes was also found to correlate to eNO levels. Infants living in the small homes $<75m^2$ ($n=9$) had significantly higher eNO levels than children living in larger homes $\geq100m^2$ ($n=22$), median 18.9 and 10.5 respectively, $p=0.01$, figure 7. These finding are not independent, there are ties between location and size. It was more common to live in a large home in the rural area than in the city as illustrated in figure 8.

**eNO in mothers**

The median eNO value of healthy mothers was 10.0 and in mothers with self-reported asthma and/or allergic rhinitis 16.0, which is significantly higher ($p=0.01$), figure 9. There was no sign of correlation between eNO values in the infants and their mothers. There was a similar non-significant trend between mother eNO levels and housing location, data not shown.

Analysing eNO levels without the allergic mothers gave too few observations to show any statistical differences.
Discussion

The main findings

Sampling of eNO using the offline tidal breathing technique with uncontrolled flow rate can be performed in infants. The procedure could be incorporated in ordinary health care screening program for children without any major disturbance. The problem for the SELMA study is more on a practical level with cost of many facemasks with the non-re-breathing valves and logistic problem in transporting NO-filled balloons for analyses. Another problem is that the procedure can be user dependent. In this study the first author (DEM) performed 69% of the measurements. The analysis of difference between users was not done owing to too few measurements from the two other authors.

There was data in support that housing’s location and size have an influence on eNO levels in infants. Infants in rural large homes had lower eNO. The fact that both, location and size had a similar statistically significant trend disputes the probability of merely a random finding. Size and location were closely related and they can both be markers of an allergy related indoor chemical factor. There was, however, no other housing factor, i.e. flooring material, type of housing or age of house that showed any significant correlation to eNO values. This might be due to many choices in the questionnaire and too few participants or a degree of uncertainty of responders of which materials the house was build of.

The significantly higher eNO levels in infants living in the centre of the city than in ones living in the rural area could alternatively be explained by the outdoor environment with air pollutant like traffic emissions, as found in a number of studies (16, 17). Housing size could also be a proxy for social factors not assessed in this study.

The lack of correlation between mothers and child eNO was unexpected but can partially be explained by statistics, the variance of the measurements is larger than the correlation coefficient. As a simile the possible signal of correlation drowns in the noisy variance background.

Problems with the method

The participation rate of 42.5% was lower than expected and technical problems with the NO analyzer hampered the expected number recruited for this report. We observed that the willingness to participate was seasonally dependent. During the summer the response rate fell compared to measurements in the spring. We just accepted if mothers did not want to participate and we did not have any structured questionnaires concerning their refusal. Most parents, who refused reported lack of interest in the study or were concerned for problems with collecting urine in their children. However parents who participated said not to experience any problems with urine collection. The problem with participation rate will be addressed differently in SELMA by personal communication with the participants prior to measurements.

The ambient NO at the time of measurements was always less than 5 ppb. Therefore the influence on eNO by atmospheric NO was considered as minimal and no measurements were disqualified due to high ambient NO levels.

The consecutive recruitment of infants gave no bias regarding age but a selection towards more girls.

Exhaled NO levels were independent of gender and age in this study, which is in accordance with many other studies (18–20), while other authors have reported that eNO increases with infants age (21, 22). This study had a narrow age range, which makes correlation estimations difficult. The observed gender bias should not influence the relevance of the statistical inference drawn from the sample.

The offline tidal breathing method of eNO assessment in young children used in the present study reflects the mixed oro-nasal eNO concentration. Some studies have reported that nasal NO elevates significantly eNO levels in mixed breath (10), while other do not report any differences between nose and mouth levels (12). Regardless of any possible difference in levels, the oro-nasal eNO differentiates in a similar way between infants with different airway diseases as eNO measured pure orally (10).
A problem with the present tidal breath off-line method is that eNO concentrations could be lowered during high expiratory flow. Total NO output could be assessed through simultaneous recording of flow and eNO. In a study in newborns showing correlation between smoke exposure and elevated eNO levels, NO output was no better parameter than eNO to distinguish between the groups (23). This implicates that it might not be of value to correct for flow. The most obvious problem in using the method in a standardized way is to get the child to breathe calmly. The results from this paper motivate further recruitment of mothers and children to obtain a larger sample for statistical analysis. When a sufficient sample is reached the inflammatory markers in urine will be analysed. This gives a better possibility to assess which inflammatory marker in infancy that will be used in a longitudinal design, in order to better understand the etiology of allergic diseases.

Acknowledgments

- Professor G.J.A Strous, the Dutch coordinator of scientific projects University Medical Centre Utrecht, for giving me the opportunity to perform my last study project in Sweden
- Dr C.K. van der Ent, Dept of Pediatric Pulmonology, University Medical Centre Utrecht, Utrecht, the Netherlands, for his support and advise
- Dr Carmelo Gabriele, Generation R study group, Erasmus Medical Centre, Rotterdam, the Netherlands for cheerful help and hands on training in doing eNO measurements in children
- Dr Mariëlle Pijnenburg Erasmus Medical Centre, Rotterdam, The Netherlands for her support and scientific advise
- Professor Kjell Alwing for his scientific advise
- Dr Anna-Carin Olin, Gothenburg University for generously lending us the offline NO analyzer
- Doctoral student, Malin Larsson and associate professor Carl-Gustaf Bornehag Karlstad University for providing sanitary towels
- Fredrik Lundin, Statistician, for statistical advice
- Margaretha Warnqvist Primary Care Research Unit for practical assistance
- The primary health care centres in Värmland, in particular the nurses in child health care at Kronoparken, Gripen, Rud and Filipstad
- County council of Värmland for the financial support from the foundation for Medical Research
- Medical engineer Mårten Mellstig, University hospital of Örebro, for kindly manufacturing the adaptor to the Mylar balloons
- Dr Torbjörn Kjerstadius for help with the management of the bio-bank
- Aerocrine, Stockholm, Sweden for providing the sensor and filters for measurements with the Niox Mino